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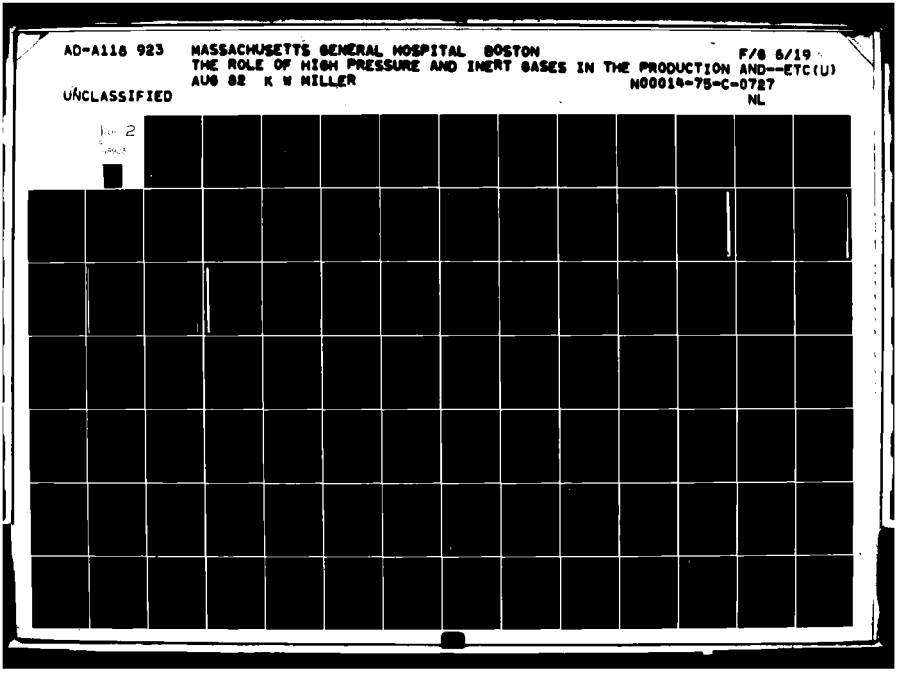

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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A118923
	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) The Role of High Pressure and Inert Gases in the Production and Reversal of the High Pressure Neurological Syndrome	5. TYPE OF REPORT & PERIOD COVERED Final, 1975-1982
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Keith W. Miller	8. CONTRACT OR GRANT NUMBER(s) N00014-75-C-0727
9. PERFORMING ORGANIZATION NAME AND ADDRESS Massachusetts General Hospital Boston, MA 02114	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS Physiology Program (Code 441NP) Office of Naval Research 800 North Quincy Street Arlington, VA 22217	12. REPORT DATE 6 August 1982
	13. NUMBER OF PAGES 11 + Reprints
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	15. SECURITY CLASS. (of this report)
	15a. DECLASSIFICATION DOWNGRADING SCHEDULE

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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

High pressure neurological syndrome, inert gas narcosis, deep diving, critical volume hypothesis

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

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A large extension of the safe diving limits can be achieved, but finally a point is reached when the HPNS can only be further postponed by adding anesthetic levels of the second inert gas. Thus, there now exists a trimix barrier to yet deeper diving.

The gas mixtures required to prevent the HPNS can be calculated using a simple model of their mechanism of action called the critical volume hypothesis.

These and further studies indicate that the several phases of the HPNS have separate etiologies and it is possible to selectively modify with drugs each of these end points. Neurochemical studies show promise of providing a deeper understanding of the underlying mechanisms.

FINAL TECHNICAL REPORT

THE ROLE OF HIGH PRESSURE AND INERT GASES IN THE PRODUCTION AND REVERSAL OF THE HIGH PRESSURE NEUROLOGICAL SYNDROME

Contract No. N00014-75-C-0727

Dates: 1 April 1973 - 31 March 1982

Principal Investigator: Keith W. Miller, M.A., D.Phil.

Departments of Anesthesia and Pharmacology

Harvard Medical School

Massachusetts General Hospital

Boston, MA 02114

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Abstract

The ability of narcotic or anesthetic gases when added to oxy-helium breathing mixtures (trimix) to increase the depth limit imposed by the high pressure neurological syndrome (HPNS) has been investigated. Five such gases all gave good protection with potencies related to their anesthetic potencies. A large extension of the safe diving limits can be achieved, but finally a point is reached when the HPNS can only be further postponed by adding anesthetic levels of the second inert gas. Thus, there now exists a trimix barrier to yet deeper diving.

The gas mixtures required to prevent the HPNS can be calculated using a simple model of their mechanism of action called the critical volume hypothesis.

These and further studies indicate that the several phases of the HPNS have separate etiologies and it is possible to selectively modify with drugs each of these end points. Neurochemical studies show promise of providing a deeper understanding of the underlying mechanisms.

Introduction

The objectives of this work were: (1) to investigate the role of high pressure and inert gases in producing and reversing the various phases of the high pressure neurological syndrome (HPNS); (2) to establish a theoretical description of the opposing effects of inert gases and pressure that would be useful in predicting gas mixtures for diving; (3) to investigate the molecular mechanisms underlying the opposing actions of pressure and inert gases; and (4) to investigate the neurochemical basis of these effects.

The role of inert gases in ameliorating the HPNS in mice

A pressure chamber was constructed to enable large numbers of rodents to be studied under well controlled physiological conditions at pressures up to 300 atmospheres (10,000 FSW). The ability of helium pressure to reverse inert gas narcosis was established by measuring the anesthetic potency of five inert gases as a function of pressure. The ability of these five gases to raise the threshold pressures at which helium compression causes various phases of the HPNS was studied similarly. The HPNS end-points studied were tremors, clonic and tonic convulsions and death. All five anesthetic gases caused marked elevations in HPNS thresholds for all end points at partial pressures below those causing anesthesia. However, to prevent the HPNS, the partial pressure of the inert gas had to be increased faster than the ability of the increased pressures thus made possible to reverse the anesthetic effect. This eventually imposes a limit on the elevation of the pressure of onset of the HPNS, because eventually anesthetic levels of the second inert gas are required. Indeed, at extremely high pressures, animals were observed to convulse even when anesthetized.

Thus, the addition of anesthetic gases to helium can significantly extend the depths to which HPNS-free dives can be achieved. However, even using optimal mixtures, the depth limit cannot be extended indefinitely. Thus, in addition to the depth limitations of air diving and of oxy-helium diving, one may now recognize a limitation to trimix diving.

One further question of interest is whether the effects of helium in reversing anesthesia and in precipitating the HPNS are the same as those of mechanical pressure itself or include a weak hidden anesthetic component resulting from the gas's slight solubility in lipid. Experiments in aquatic animals were able to

reveal unequivocally the anesthetic effect of helium and to separate it from the effects of mechanical pressure.

Theoretical calculations

The data on HPNS thresholds obtained above were used to test the hypothesis that a given HPNS threshold is achieved when some hydrophobic region in neural tissue is compressed beyond a critical amount by application of hydrostatic pressure. The absorption of inert gases into this hydrophobic region causes a compensating expansion which raises the threshold pressure for the HPNS. (The critical volume hypothesis.)

Calculations were performed using the known physical properties of the inert gases to test this hypothesis. Good agreement with the data was obtained for each of the different phases of the HPNS. The model predicts, however, that each end-point is mediated by a separate site with distinct physical properties. Using this model it is possible to calculate the gas mixtures which will keep each site close to its optimal volume and so prevent the expression of symptoms. The gas mixtures required are a function of depth. Because each of the sites of action have different physical properties, they require different gas mixtures for a constant volume titration. Gas mixtures can be chosen which prevent all sites from exceeding their critical volume, but the constraints increase with pressure. At higher pressures, the range of gas mixtures that can be successfully employed becomes smaller and eventually vanishes (i.e. no mixture will prevent all symptoms).

Molecular level studies

The hypothesis that the HPNS might be caused by a decrease in lipid fluidity, and therefore prevented by drugs which fluidize membranes, was investigated.

All volatile and gaseous anesthetics and alcohols were found to fluidize membranes regardless of the membrane's composition. This was consistent with their ability to antagonize all phases of the HPNS. However, many intravenous anesthetics and tranquillizers were found to be highly selective as membrane fluidizers. In particular, many caused membranes low in cholesterol to be ordered rather than fluidized, but all drugs fluidized membranes high in cholesterol. This suggested that some of these agents might have selective anti-HPNS actions independent of their sedative effects.

The role of various pharmacological agents in HPNS

A number of agents were chosen, based on the above studies, and their anti-HPNS profiles studied. All agents which had anesthetic properties (e.g. phenobarbital, urethane) protected against all phases of the HPNS in proportion to their anesthetic potency. Other agents protected against some, but not all, aspects of the HPNS, and others (e.g. phenytoin) exacerbated some phases of the HPNS, protected against others and were ineffective against yet other end points.

These results were consistent with the broad anti-HPNS effectiveness of inert gas anesthetics and of the heterogeneous origin of the different HPNS endpoints. They also suggest that selective pharmacological control of each phase of the HPNS is possible, and that more detailed neuropharmacological studies may enable the depth limitations inherent in the use of trimix to be overcome.

Neurochemical studies

Few neurochemical studies have ever been performed under high pressures of diving gases. An apparatus to enable the binding of neurotransmitters to their receptors was developed. Helium pressure was found to decrease the binding of acetylcholine to the nicotinic receptor in electric tissue of rays by decreasing the binding affinity without altering the number of receptor sites. The anesthetic diving gases, however, had the opposite effect. Thus, this system appears to reflect some of the properties of these gases seen in vivo and may offer deeper insights into the underlying etiology of the HPNS. Further neuropharmacological studies may thus lay the foundation for safe diving at depths beyond the trimix barrier.

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SESSION IX
NARCOSIS AND HPNS

68 AMELIORATION OF THE HIGH PRESSURE NEUROLOGICAL SYNDROME (HPNS) BY ANESTHETIC GASES. R.A. Smith and K.W. Miller. Harvard Medical School Department of Anesthesia and Pharmacology, Massachusetts General Hospital, Boston, MA. 02114.

The HPNS is attenuated by admixture of nitrogen to the breathing gases in mice and man. We have examined a wide variety of gases (N_2O,N_2,Ar,CF_4 and SF_6) and tested the critical volume hypothesis applied to the HPNS. For anesthetic action this hypothesis asserts that anesthesia occurs when hydrophobic regions of membranes are expanded beyond a critical volume. Modified to apply to the HPNS it states that at a given behavioral endpoint (e.g. convulsions) the hydrophobic site mediating that effect becomes compressed beyond a critical volume. Addition of an anesthetic would expand the site and offset the compression. In each case we determined the median pressures for appearance of complete body spasms, clonic and tonic convulsions and for death not associated with a tonic convulsion. Each gas was studied with 10 animals per dose at three to five doses. Anesthetic gas was admitted first followed by helium. Helium alone was used in control runs. Compression rates were constant at 1 atm min^{-1}. The median pressures for all of the HPNS effects were increased by all anesthetics. To test the critical volume hypothesis we first determined the solvent model which best represented the hydrophobic site mediating each endpoint. These solvents were used to calculate the compressibilities of these different sites. These were ($x10^{-5}$ atm^{-1}): 12 for complete spasms (carbon disulfide); 4.5 for tonic convulsions (carbon disulfide); 16 for clonic convulsions (cyclohexane) and 12 for death not associated with tonic convulsions (benzene). These compressibilities range from approximately two to eight times that for the anesthetic site ($\sim 2.1x10^{-5}$ predicted from carbon disulfide). This suggests that the physical properties of the site for different endpoints are different and that agents which selectively expand a given site may prevent its compression and thus alleviate the associated syndrome.

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AN OPIOID PEPTIDE WITH GENERAL ANESTHETIC PROPERTIES. Barbara A. Dodson* and Keith W. Miller. Depts. of Anesthesia and Pharmacology, Mass. General Hospital, Harvard Medical School, Boston, MA 02114.

Many structural classes of anesthetics are pressure reversible. This has yet to be reported for the new peptide anesthetics. One such peptide anesthetic is BW871C (Tyr.D-Ala.Gly.Phe.D-Leu.NMEr.HCl, Burroughs-Wellcome), a naloxone reversible, anti-nociceptive leucine-enkaphalin analog reported to cause loss of righting reflex (LRR) in rodents (Br. J. Pharm. GR, P. 159 (1980)). We found the peptide's ED_{50} for LRR in tadpoles increased from 23 ± 1.6 mM at 1 atm to 44 ± 6.5 mM with 120 atm of helium, a percentage increase similar to that found for the volatile anesthetic octanol, thus demonstrating conventional pressure reversibility. The peptide's octanol/water partition coefficient of 2187, as calculated by Hansch analysis, compared well with the value predicted by the Meyer-Overton solubility hypothesis for other classes of general anesthetics. However, unlike octanol, the peptide's anesthesia was reversible with naloxone, but only at high doses (10^{-4}M). One possible explanation for our findings is that the peptide's in vivo effects depend on both its lipid solubility and its specific opiate receptor interaction. Reversal of either of these components would then lead to a reversal of anesthesia. (Supported in part by ONR contract #N00014-75-0727 and NIH Training Grant #GM-07592-03.)

7893

IS HELIUM PRESSURE EQUIVALENT TO HYDROSTATIC PRESSURE: AN IN VIVO TEST OF THE CRITICAL VOLUME HYPOTHESIS. Z.W. Janowski, R.A. Dodson*, and K.W. Miller, Depts. of Anesthesia and Pharmacology, Mass. General Hospital, Harvard Medical School, Boston, MA 02114.

Helium is often used in lieu of hydrostatic pressure (HSP) in in vivo studies of the effect of pressure. As predicted by the Critical Volume Hypothesis, we found a significant difference in the ability of HSP versus helium in pressure reversing urethane anesthesia in tadpoles: helium was only 70% as effective as HSP at isobaric conditions of 110 atm. (Urethane ED_{50} at 1 and 110 atm were 13.6 ± 0.69 mM and 19.8 ± 0.7%, respectively, for helium, as compared to 13.6 ± 1.3 mM and 28.4 ± 1.4 mM for HSP.) Helium's predicted latent anesthetic component was shown with the effect on urethane anesthetized tadpoles by isobarically switching from HSP to helium. At 110 atm the LRR increased from 50% to 80% with the switch to helium. An explanation for this component was found in the attenuating ability of other inert gases to pressure reverse anesthesia. The attenuation was inversely proportional to the gas's lipid solubility ($r = 0.985$). Our experimental values compared well with those derived for model membranes using the hypothesis. These findings support the hypothesis as a theoretical model for predicting the physicochemical actions of anesthetics in membranes. (Supported in part by ONR contract #N00014-75-C727 and NIH Training Grant #GM-07592-03.)

25 THE IN VIVO EFFECTS OF DIFFERENT PRESSURIZING AGENTS. Barbara A. Dodson*, Zygmund W. Furmaniuk, Jr.*, and Keith W. Miller. Depts. of Anesthesia and Pharmacology, Mass. General Hospital, Harvard Medical School, Boston, MA 02114.

Studies of the effects of pressure on biological systems must be interpreted with respect to the pressurizing agents used. We examined the abilities of helium, hydrogen, neon, nitrogen, argon and mechanical compression (hydrostatic pressure, HSP) to pressure reverse urethane anesthesia in tadpoles. We also compared the effects of the pressurizing agents on the animals without anesthesia. HSP caused the greatest degree of pressure reversal with a doubling of urethane's ED_{50} with 110 atm of pressure. HSP alone caused hyperactivity (HPNS) leading to LRR at an EP_{50} (effective pressure) of 137 atm. At 110 atm helium was only 70% as effective as HSP in reversing urethane anesthesia and itself had an EP_{50} of 172 atm. Neither hydrogen nor neon caused much change at 110 atm on urethane's ED50, and themselves had little effect at pressures of less than 200 atm. Both argon and nitrogen were themselves anesthetic with ED_{50}'s of 75 and 42 atm respectively, and added to, rather than reversed, urethane anesthesia. From our results, it appears that the ability of a pressurizing agent to reverse anesthesia is inversely proportional to the agent's lipid solubility. These results also suggest that neon and hydrogen, with their lack of effects at high pressures, may prove to be more useful than helium alone in avoiding both narcosis and HPNS in deep-sea divers. (Supported in part by ONR contract #N00014-75-0727 and NIH Training Grant #GM-07592-03.)

Reprinted from
6 September 1974, Volume 185, pp. 867-869

SCIENCE

Inert Gas Narcosis, the High Pressure Neurological Syndrome, and the Critical Volume Hypothesis

Keith W. Miller

Inert Gas Narcosis, the High Pressure Neurological Syndrome, and the Critical Volume Hypothesis

Abstract. *The hypothesis that general anesthesia or pressure-induced convulsions occur when a hydrophobic region is expanded or compressed, respectively, by critical amounts is consistent with recent data obtained with mice. Calculations show that anesthesia occurs at an expansion of 1.1 percent and convulsions at a compression of 0.85 percent, the latter site of action being more compressible.*

The replacement of nitrogen by helium as the inert gas diluent in deep-diving breathing mixtures has removed the constraint of nitrogen narcosis (*1*), and simulated depths of 600 m (2000 feet, 61 atm) have been reached recently in France. However, a new barrier to deeper diving is the high pressure neurological syndrome, a hyperexcitability which first manifests itself in man at about 20 atm as a coarse tremor of the limbs (*2*). At higher pressures (60 to 100 atm) convulsions occur in experimental animals, including primates, and manned diving programs have consequently adopted cautious compression schedules (for example, 7½ days in the 600-m dive). Addition of narcotic gases to the breathing mixture has an ameliorating effect in animals (*3*).

Pressure reversal of anesthesia is another example of an effect of pressure on the central nervous system, and has led to the formulation of the critical volume hypothesis (*4*). This states that anesthesia occurs when the volume of a hydrophobic region is caused to expand beyond a certain critical volume by the absorption of an inert substance. An applied pressure opposes this expansion and reverses anesthesia. In this report, it is proposed that the hypothesis may be extended to include the high pressure neurological syndrome by assuming that convulsions occur when some hydrophobic region has been compressed beyond a certain critical amount by the application of pressure. Absorption of an inert gas will compensate for such compression and raise the convulsion threshold pressure. This extension of the critical volume hypothesis has the attraction of offering a unified description for the interaction between pressure and narcotic gases in the central nervous system with respect to hyperexcitability and anesthesia. It could also provide a theoretical foundation for the use of inert gas mixtures in deep diving. The few studies that have been made of the effect of anesthetics and pressure on simple membranes suggest that the hydrophobic region is membranous in nature (*5*, *6*).

The fractional expansion, E, that occurs when a gas at a partial pressure, P_a, dissolves in a bulk solvent is given by

$$E = \bar{V}_a x_a P_a / \bar{V}_m \qquad (1)$$

where $\bar{V}_a$ is the partial molar volume of the gas in the solvent of molar volume $\bar{V}_m$, and x_a is the mole fraction solubility of the gas in that solvent when its partial pressure is 1 atm. In addition, physical compression of the liquid occurs according to its compressibility, β, and the total pressure, P_T (fractional compression $= \beta P_T$). In fact, for the less soluble gases, helium and neon, the compression term is larger than the expansion term and net compression results; hence they are not anesthetics. For the more soluble anesthetic or narcotic gases, such as N_2, Ar, and N_2O, net expansion occurs (*4*). Equation 1 must be corrected for gas imperfections and for the slight dependence of solubility on total pressure. The nature of these corrections has been given in a previous paper (*4*), in which the critical volume hypothesis accounted for pressure reversal of anesthesia data for newts. Here, the treatment will be applied to mammals.

Quantitative data for the pressure reversal of anesthesia in mice are available for three gas mixtures—He : N_2O, Ne : N_2O, and H_2 : N_2O (*7*)—while comparable data for the elevation of convulsion threshold are available for He : N_2, He : N_2O, and He : H_2 (*3*). The study of the high pressure neurological syndrome is complicated by the apparent dependence of the convulsion threshold on the strain of mice used and, to some extent, on the compression rate employed. While these variations deserve more detailed investigation, they are not large, and the data used in this study are internally consistent, having been obtained in one laboratory by a standardized procedure.

The expansion caused by dissolution of the inert gases (Eq. 1) was calculated for the experimental isonarcotic and isoconvulsion end points. This is shown in Fig. 1 as a function of pressure for the model solvent benzene. Such a plot should yield a linear relation where the slope gives the compressibility of the site of action and the intercept gives the critical volume

Table _____ of calculations according to the critical volume hypothesis for three solvent models for the pressure reversal of anesthesia (*8*) and the high pressure neurological syndrome (convulsions) (*3*) in mice. Physical parameters for these calculations have been given previously (*4*); in addition, the solubility of neon in carbon disulfide is 4.8×10^{-4} mole fraction (*17*). For hydrogen $\bar{V}$ was taken as 35 ml/mole for all solvents (*18*). The Bunsen partition coefficient of hydrogen in olive oil was 0.04 (*9*). The compressibilities of olive oil, benzene, and carbon disulfide are 6, 9, and 7×10^{-5} atm^{-1}, respectively (*4*). For the critical volume change and compressibility, values are means ± standard deviations.

Solvent	Effect	Critical volume change (%)	Compressibility ($\times 10^5$ atm^{-1})	Correlation coefficient
Olive oil	Anesthesia	+0.35 ± 0.03	3.2 ± 0.56	.85
	Convulsions	−0.39 ± 0.12	7.1 ± 1.20	.91
Benzene	Anesthesia	+1.1 ± 0.02	3.0 ± 0.39	.91
	Convulsions	−0.85 ± 0.14	13.9 ± 1.37	.96
Carbon disulfide	Anesthesia	+0.60 ± 0.04	3.8 ± 0.68	.84
	Convulsions	−0.60 ± 0.16	10.0 ± 1.59	.92

change required for anesthesia or convulsions at 1 atm absolute. Results of the calculations for three solvents, which experience shows are good analogs of the anesthetic site (*8*), are summarized in Table 1. All three model solvents produce a good fit of the data for both anesthesia and convulsions.

The most striking conclusion from Table 1 is that a particular model solvent gives a self-consistent description in terms of the critical volume hypothesis for the volume changes associated with anesthesia and convulsions; that is, a particular positive or negative change in volume at the sites of action is critical and results in profound effects in the central nervous system. The predicted percentage expansions vary somewhat depending on the solubility and molar volumes of the solvents. The compressibilities are close to those observed experimentally in each case, although the site mediating convulsions is two to five times the more compressible of the two, indicating that two separate sites of action exist for anesthesia and convulsions. Further discussion of the differences between the solvents seems unlikely to be profitable, and it would be more interesting to know the results of such calculations for real membranes, but the few physical data available (*9*) only allow one to conclude that the volume changes given by these solvents are of the order of magnitude to be expected in real membrane systems.

A further evaluation of the physical parameters of the site of action, which is independent of gas solubility data, may be made by using results of recent experiments (*10*) in which mice breathing an oxygenated fluorocarbon fluid were compressed hydraulically to produce convulsions. These experiments were conducted at a number of reduced rectal temperatures, allowing a short extrapolation to 37°C, which gives a convulsion threshold of 62 atm at compression rates comparable to those in Table 1. If we assume that the compressibility of the convulsive site is that given by each of the model solvents (Table 1), then these data yield critical volume changes of −0.44, −0.86, and −0.62 percent for olive oil, benzene, and carbon disulfide, respectively—values in good agreement with those in Table 1. These liquid breathing experiments raise a further

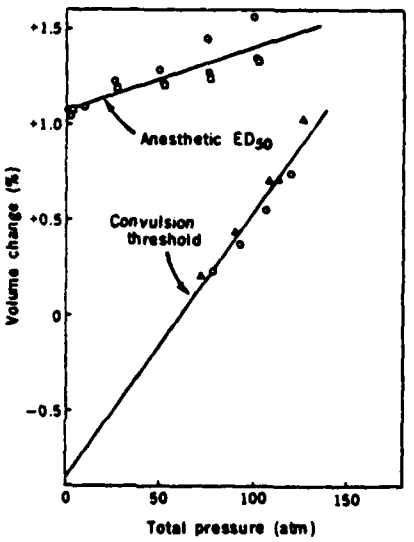

Fig. 1. Calculated expansion of benzene (Eq. 1) (*4*) caused by mixtures under isoanesthetic (*8*) and isoconvulsive (*3*) conditions at various pressures. The intercepts yield the critical volume changes, and the slopes, the compressibilities. The anesthetic ED_{50} is the dose effective in anesthetizing 50 percent of a group of animals.

intriguing possibility. Liquids that have been compressed to remove all gas nuclei may be subjected to negative pressures of several hundred atmospheres without cavitation (*11*). Would a similarly treated mouse thus be anesthetized by negative pressures of the order of 50 to 100 atm?

For diving practice, the unified critical volume hypothesis suggests that the composition of the breathing mixture should be adjusted so as to produce no volume change at the site of action. Reversal of nitrogen narcosis by helium pressure has been observed in man (*12*), while the amelioration of the high pressure neurological syndrome in divers by adding anesthetic gas to their breathing mixtures is the object of active investigation (*3*). However, since the site for convulsions appears to be two to five times more compressible, it is clearly not possible to completely prevent volume changes at both sites by titrating the inert gas against the absolute pressure. Nonetheless, minimization of the changes should enable divers to maintain performance levels at considerably greater depths than those they currently achieve breathing helium-oxygen. Ultimately, it should be considered that the sites of action referred to here may only be the most sensitive of a spectrum of sites, as is suggested by the respiratory and cardiac problems encountered in mammals above 100 atm (*13*). This possibility, together with the different compressibilities in Table 1, suggests that more specific pharmacological intervention will eventually be required if man is to achieve depths of ever greater magnitude.

The success of the critical volume hypothesis in providing a self-consistent explanation of the interaction of anesthetic gases and pressure is rather remarkable. Although it cannot be ruled out that such success arises by chance, the hypothesis provides specific predictions about the sites of action which are accessible to experimental tests at a biophysical level. It seems, at present, most probable that the sites of action are situated in the lipid bilayers of some membranes (*14*). This interaction in itself is probably not directly responsible for the effects observed; rather, one might suppose that the membrane perturbations influence the functions of some membrane proteins in the neurological apparatus. Evidence for such a view may be found in stud-

ies of the red blood cell membrane (*5*), rat phrenic nerve (*15*), and the behaviors of simple antibiotic ionophores, such as valinomycin (*6*, *14*) and gramicidin (*16*). Measurement of the appropriate membrane properties should enable this interpretation of the critical volume hypothesis to be examined in more detail.

KEITH W. MILLER

Harvard Medical School, Departments of Pharmacology and Anesthesia, Massachusetts General Hospital, Boston 02114

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3 June 1974

Review of the effects of pressure and anaesthetics on membranes

K. W. Miller

The fluid mosaic model of membrane structure envisages proteins "floating" on, or in, a lipid bilayer (1). At the moment we know little about the nature of the membrane proteins. However, anaesthetics may interact with the lipids, producing a perturbation which in turn affects protein function. This hypothesis implies that the anaesthetics do not have specific sites of interaction but instead dissolve in the fluid bilayer in an analgous manner to dissolving in a bulk fluid. The membrane increases in volume when anaesthetics dissolve in it, and decreases in volume when pressure is applied. Evidence for this has been obtained from experiments with monomolecular layers of lipids at a water-gas interface in which the film pressure at constant area (related to the area at constant film pressure) increases in the presence of argon and nitrogen but decreases in the presence of helium (which has a minimal anaesthetic effect)(2). Similar changes in surface area with other drugs have also been shown in red blood cells (3). The increase in volume is not the same in all directions and it has been demonstrated that anaesthetics increase the surface area while at the same time decrease the thickness of a bilayer (4).

These volume changes can be related to the type of functional changes which may be important in neural function, such as the movement of ions across the membrane. One suitable model system is a lipid bilayer with an ionophore which acts as an ion carrier across the membrane. When the anaesthetic is added the ion flux increases as the membrane fluidity increases and the anaesthetic

concentration causing this effect can be correlated with that blocking conduction in the frog sciatic nerve. Pressure alone reduces the ion permeability and it is possible to balance the effects of anaesthetics and pressure, with the permeability returning to the control value. This effect is not simply the displacement of the anaesthetic from the membrane under pressure, since it does not matter at which stage the anaesthetic is added. It is possible to calculate the increase in volume of the membrane caused by the anaesthetics and the compressibility of the system when pressure is applied. The calculated compressibility (2×10^{-5} atm^{-1}) is consistent with that expected for hydrocarbon compressibility (5).

When anaesthetics dissolve in the lipid bilayer the volume increases by about 0.25%. The effect of this volume change may be magnified by several factors. First, the proportional increase in surface area of the bilayer may be up to ten times greater than the volume increase. Second, the volume change may alter phase transitions in membranes. One phase transition is when the arrangement of the lipids changes from a more solid gel phase to the more liquid fluid phase. An analogy can be made with a melting point which is usually associated with an expansion of a substance. However, unlike a melting point the phase transition occurs over a temperature range up to $5^{o}C$. Interaction of anaesthetics with the membrane tends to shift the bilayer towards increased fluidity, while increased pressures have the opposite effect. Studies with model systems have examined the effects of pressure at different temperatures on either side of the phase transition. The changes observed for the same pressure increase have been greater in the gel phase than the fluid phase and very much greater in the intermediate range of the phase transition(6).

It has been postulated that such phase transitions are important in modifying protein function and it may be that this is one of the ways in which volume changes can have a profound effect on functional changes.

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Application of the critical volume hypothesis to problems of deep diving

K.W. Miller

The critical volume hypothesis offers a description of the interactions of pressure and inert gases which produce anaesthesia and convulsions. It accounts for the pressure reversal of anaesthesia and anaesthetic antagonism of pressure induced convulsions (HPNS). The hypothesis may be stated as follows: anaesthesia occurs when the volume of a hydrophobic region is caused to expand beyond a certain critical amount by the absorption of an inert substance; an applied pressure opposes the expansion and reverses the anaesthesia. Conversely, convulsions occur when some hydrophobic region is compressed beyond a certain critical amount by application of pressure; absorption of an inert gas will compensate for such compression and raise the convulsion threshold pressure. Studies on the pressure reversal of anaesthesia(1) and the convulsion threshold in mice (2) provide a basis for testing the hypothesis.

The percentage expansion, Eg. caused by a dissolved gas, is given by :

$$Eg = \overline{V}_2 \cdot x_2 \cdot Pa/Vm$$

Where $\overline{V}_2$ is the partial molar volume of the gas in the solvent of molar volume, Vm, x_2 its mole fraction solubility and Pa the applied partial pressure. In a gas mixture each gas contributes additively and at elevated pressures compression must be included to give the net volume change E_T.

$$E_T = \left[\sum_i \bar{V}i \cdot xi \cdot Pai/Vm\right] - \beta P_T$$

or $$E_T = Eg - \beta P_T$$

where β is compressibility and P_T total pressure. A set of isonarcotic (or isoconvulsive) data at different pressures, together with $\bar{V}_2$, x_2 and Vm data from a model solvent, allow Eg to be calculated at each P_T. A plot of Eg . vs P_T then yields the compressibility (slope) and critical percentage expansion (intercept). Figure 1 shows such a test. β for the convulsive site is greater than for the anaesthetic site, whilst critical volumes for these two sites are of the same magnitude but opposite in sign.

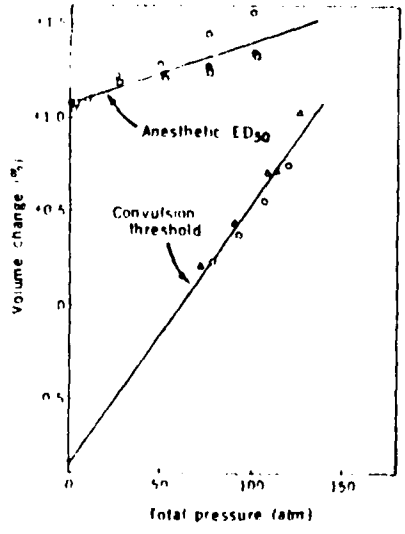

Figure 1

Knowing these physical parameters of the sites of action we can calculate the pressure at which convulsions or anaesthesia will result for any given gas or gas mixture. (See Figure 2). Carrying out these calculations for mixtures enables one to construct a graph or map which predicts safe mixtures at various pressures (Figure 3).

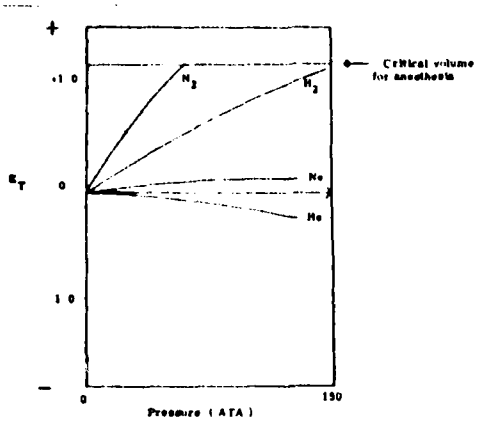

Figure 2

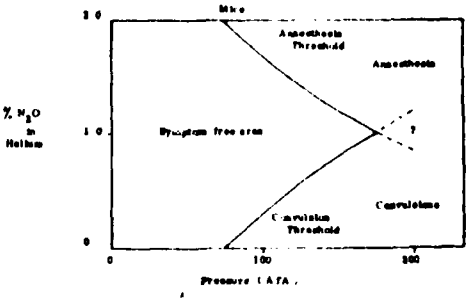

Figure 3

Preliminary application to manned diving was made for He - N_2 mixtures. Deriving the critical volume and β for narcotic and convulsive sites in man is difficult because of the paucity of quantitative data. However, tentative predictions suggest that inclusion of <u>not more</u> than 15% N_2 should avoid narcosis at all pressures up to the convulsion threshold. Considering the current uncertainties in the calculations, 10 $\pm$ 5% N_2 may be regarded as the best working mixture. The convulsive threshold itself is significantly raised by use of such mixtures compared to values for pure helium. The theory allows ready extrapolation to other gas mixtures. The immediate need is for more data at high pressures on the narcotic threshold of gas mixtures. Ideally the similar data for convulsion threshold is required, but this may be obtained from experiments on primates to a good approximation.

The critical volume hypothesis provides a semi-empirical theoretical approach which enables optimum diving gas mixtures to be predicted.

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(Reprinted from Nature, Vol. 263, No. 5574, pp. 253–255, September 16, 1976)

General anaesthetics can selectively perturb lipid bilayer membranes

THE circumstantial evidence that anaesthetics act primarily by increasing the fluidity of membranes is quite strong. The gaseous, volatile, barbiturate, steroid and alcohol anaesthetics have all been shown to fluidise phosphatidylcholine–cholesterol lipid bilayers and the demonstration has also been made for some biological membranes[1–4]. Furthermore, a number of lipophilic substances, such as the higher alkanols, do not fluidise membranes and are not anaesthetics[3]. In some cases, a correlation between nerve-blocking potency and the action of anaesthetics in perturbing lipid bilayers has been observed[5]. Moreover, pressure counteracts the fluidising effects of anaesthetics just as it antagonises general anaesthesia *in vivo*[6–9]. The overall success of the fluidised lipid hypothesis tends to be its major drawback, for if anaesthetics fluidise membranes indiscriminantly then the hypothesis fails to provide a unique mechanism for their selective depression of neuronal function. We show here that lipid composition may modulate the ability of an anaesthetic to fluidise membranes more than has been generally supposed.

The effect of anaesthetics on membrane fluidity has been studied by intercalating fatty acids, or phosphatidylcholine (PC), labelled with a nitroxide reporter group into lipid bilayer membranes of various compositions. The anaesthetics studied showed wide variations in their ability to fluidise phospholipid bilayers. Results and experimental conditions are outlined in Table 1. All anaesthetics studied fluidised PC bilayers low in phosphatidic acid (PA) and high in cholesterol (Chol) (4%PA: 33%Chol) as evidenced by the consistent decrease in order parameter. At one extreme, halothane and urethane fluidised all the membranes studied, whereas at the other, pentobarbital and alphaxalone fluidised only those with 4%PA : 33%Chol. Increasing cholesterol content conferred fluidising ability on all anaesthetics, but increasing the negatively charged PA tended to confer an ordering ability in some cases. A more detailed examination of the effects of lipid composition was made with pentobarbital and octanol (Table 2). The effect of pentobarbital switched from fluidising to ordering when, in the 4%PA : 33% Chol membranes, either the cholesterol content was lowered to 5–10% or PA was increased from 4 to 10%. Octanol always fluidised.

Our results in 4%PA : 33%Chol membranes are consistent with previous studies which have been carried out primarily in membranes of this cholesterol content, which is typical of nerve[2–4]. There are two isolated reports in the literature that halothane[11] and pentobarbital[12] order phospholipid membranes, as do local anaesthetics[18].

Spin-label[13] and deuterium magnetic resonance[14] studies suggest that the acyl groups of phospholipids are tilted near the head group region. General anaesthetics might reduce this tilt leading to a decrease in packing density as has been reported for tetracaine[13]. If so, the ordering effect should be weaker deeper in the bilayer and preliminary results with PC labelled with 8-doxylstearic acid at the β position are consistent with this explanation. In this context it is interesting to note that the three anaesthetics which increase anisotropy in the 4% PA bilayer all have structures which include rigid rings. A complete physical explanation of our results must await more detailed study, however.

The most important pharmacological aspect of this work concerns the specificity of action of anaesthetics. Although they are regarded conventionally as nonspecific drugs, many membrane processes are unaltered at anaesthetic doses. Thus the Na^+/K^+ ATPase of red blood cells[15] and synaptosomes[16] is unaffected at high, almost lytic, doses. This specificity might, *a priori*, reside in the primary perturbation of the lipid as well as in the secondary reaction of a given membrane protein to that lipid perturbation. Our work shows that the primary perturbation can no longer be thought of in terms of the lipid solubility of an anaesthetic alone; it is necessary to introduce the concept of fluidising efficacy, which we define as the rate of change of membrane fluidity with the concentration of anaesthetic in the membrane. Defined thus, fluidising efficacy need not be independent of anaesthetic concentration: thus Rosenberg reported unequivocal biphasic effects of halothane at physiological concentrations in palmitoyllauroyllecithin bilayers[11]. Most studies so far, however, have shown linear effects[2,3] or only weak nonlinear ones[4]. Three of the anaesthetics we examined could both order or fluidise bilayers, depending on their lipid composition, and thus indubitably exhibited both negative and positive fluidising efficacy; whereas for halothane, octanol and urethane, the fluidising efficacy was positive in all the membranes studied. For the latter anaesthetics we are unable to tell if the magnitude of the efficacy varies from membrane to membrane because the membrane solubilities are unknown. Our conclusions are thus based on the anaesthetics where a change in sign of the order parameter was observed.

To be consistent with the fluidised lipid hypothesis, anaesthetics should exhibit a positive fluidising efficacy at their site

Table 1 The change in order parameter, ΔS, measured by 5-doxylstearic acid in lipid bilayer membranes exposed to anaesthetic agents

Anaesthetic	Concentration	Bilayer composition, balance PC		
		4%PA	20%PA	4%PA:33% Chol
Halothane	11 mM	−0.03	−0.03	−0.06
Urethane	90 mM	−0.08	−0.03	−0.03
n-Octanol	46 mM	−0.02	−0.06	−0.08
Ketamine	25 mM	−0.03	0	−0.03
Alphaxalone	18 mM	−0.02	−0.03	−0.02
Pentobarbital	16 mM	−0.02	−0.02	−0.03

PC, Egg yolk phosphatidylcholine; PA, egg yolk phosphatidic acid; Chol, cholesterol; halothane is $CF_3CHClBr$; urethane is ethylcarbamate; ketamine is 2-(methylamino)-2-(2-chlorophenyl) cyclohexanone and was a gift of Parke-Davis; alphaxalone is 3α-hydroxy-5α-pregnane-11,20-dione and was a gift of Glaxo. Lipids and spin labels in organic solvents were dried down together in pear-shaped flasks. Involatile anaesthetics were added in organic solvents before drying down; volatile agents were added later after the lipids had been dispersed in solution buffered at *p*H 7.0 by vortexing, and their concentration was checked by gas chromatography. Final lipid concentration was about 20–30 mg ml^{-1}, and the spin labels constituted about 1 mol % of the lipids. Anaesthetics were equilibrated with the bilayers up to 24 h before being sealed in 1-mm glass capillaries and equilibrated at 25 ± 0.5 °C in the cavity of either a Varian E-9 or E-109 electron spin resonance spectrometer operating at 9.5 GHz. Order parameters, ΔS, were calculated from spectra according to the method of Hubbell and McConnell[10]. A decrease in ΔS indicates a less anisotropic distribution of the label and a more fluid membrane. Changes in ΔS less than 0.01 were not considered significant. High doses of anaesthetics were used to obtain large changes in ΔS, which is a linear function of anaesthetic concentration within experimental error[2,3].

Table 2 The change in order parameter caused by anaesthetics in PC lipid bilayer membranes containing varying proportions of PA and Chol

Anaesthetic	PA	Cholesterol				
		0	5%	10%	20%	33%
Pentobarbital	4%	+0.02	+0.02	0	−0.02	−0.03
16 mM	10%	—	−0.05	+0.04	−0.03	−0.05
	20%	+0.02	−0.09	−0.05	−0.02	−0.03
Octanol	4%	−0.02	—	—	—	−0.08
46 mM	10%	−0.05	−0.02	−0.03	−0.08	−0.04
	20%	−0.06	—	−0.03	−0.02	−0.03

of action. In agreement with this, all six fluidised 4%PA: 33%Chol bilayers and pressure reversal of anaesthesia *in vivo* has been demonstrated for halothane[6,9], alphaxalone (as the clinical mixture althesin, which includes one third alphadolone acetate, a steroid with half the potency of alphaxalone)[9], pentobarbital[17], urethane[18] and ketamine (M. Wilson and K. W. M., unpublished). Long chain alcohols, which are not anaesthetics and do not fluidise membranes[2,19], might have lower efficacy than the short chain alcohols, or might simply have a lower membrane solubility. In the latter case, if the efficacy is about normal they might act additively with other anaesthetics as has been observed for some fluorinated hydrocarbons[20]. In all cases the magnitude of the fluidising efficacy can only be evaluated if the membrane partition coefficient is known, which is not often the case.

A corollary of the above argument is that only membrane regions where anaesthetics exhibit positive fluidising efficacy offer putative sites of action for anaesthetics. Thus, those anaesthetics, such as pentobarbital, which exhibit a positive fluidising efficacy in a restricted range of compositions might be useful for defining the nature of such sites. Our studies suggest they must have greater than 10% cholesterol and a charge density between 0.12 and 0.46 per phospholipid. This is consistent with the known compositions of neurones (30–50%Chol; 12–20% phospholipids bearing a single negative charge)[21], although our phospholipids are not typical of the variety found in biological membranes. Two factors could limit the usefulness of such an approach. First, the presence of protein in a lipid bilayer will probably exert an additional influence on the fluidising efficacy of anaesthetics–a single report that halothane orders rat brain synaptic membrane is cautionary[11]. Second, the lipids in biological membranes are heterogeneously distributed both across and in the plane of the membrane[22,23].

Large negative efficacies might also result in pharmacological effects[24]; indeed lipid ordering has been suggested as the cause of the hyperbaric convulsions exhibited by mammals at pressures in excess of 70 atm (ref. 25). We have shown that merely increasing the PA content from 4 to 10% in a 33% cholesterol bilayer changes the fluidising efficacy of pentobarbital from plus to minus. It remains to be seen how far the delicate balance between anaesthetic and convulsant activities seen with many barbiturates can be explained in this manner.

Our work has been concerned exclusively with the primary anaesthetic-induced lipid perturbation. The sensitivity of membrane proteins to such perturbations might also vary.

The support of the National Institute for General Medical Sciences and the Office of Naval Research are acknowledged.

KEITH W. MILLER
KAM-YEE Y. PANG

Departments of Pharmacology and Anesthesia, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114

Received May 17; accepted June 29, 1976.

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Printed in Great Britain by Henry Ling Ltd., at the Dorset Press, Dorchester, Dorset

The Opposing Physiological Effects of High Pressures and Inert Gases

KEITH W. MILLER

Reprinted from FEDERATION PROCEEDINGS
Vol. 36, No. 5, April, 1977

The opposing physiological effects of high pressures and inert gases[1]

KEITH W. MILLER

Department of Anesthesia and Pharmacology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114

The response of mammals to elevated ambient pressures has to be considered in the context of the physiological properties of the gases breathed. Because of the toxic properties of hyperbaric oxygen (24) an inert gas diluent is generally included in a diver's breathing mixture. The use of nitrogen in this role causes euphoria and an impairment of higher mental processes that becomes more and more serious at depths deeper[2] than 30 m (1). These symptoms of nitrogen narcosis bear some similarity to those in the early stages of anesthesia, and studies on mice show nitrogen produces complete anesthesia at a partial pressure of 35–40 atm. As we shall see, certain simple concepts of the mode of action of these lipid soluble anesthetics prove to have considerable utility in predicting the physiological outcome of breathing increased partial pressures of inert gases.

Hildebrand and co-workers suggested that helium, which is much less soluble than nitrogen, should be a superior inert gas for diving (8). Experimental chamber dives to as deep as 600 m have subsequently been made without signs of inert gas narcosis. However, a new phenomenon, characterized by trembling of the extremities, excitability and certain EEG changes, has been discovered and called the high pressure neurological syndrome (HPNS) (2). This syndrome can be relieved somewhat by slow compression rates but these themselves impose a limitation on diving practice. The HPNS appears to be a function of the elevated pressure per se and not related to inert gas narcosis since addition of nitrogen to the $He-O_2$ mixture to form a trimix ameliorates the HPNS (2). The ultimate physiological limit to deep diving thus lies beyond 60 atm and remains to be defined.

In order to consider further what limitations pressure per se may impose it is necessary to consider work on animals. The classical work of Regnard, Ebecke, Cattell and others has been reviewed recently by Fenn (5). Aquatic animals show a general stimulation of the central nervous system at pressures around 50 atm. At 200–300 atm paralysis results from spontaneous muscle contraction, while higher pressures still (400–600 atm) prove lethal. Work on amphibia enables the effect of hydrostatic and gas pressure to be compared. Newts compressed hydraulically showed slight paralysis at 140 atm and complete paralysis at 200 atm. In helium and neon paralysis occurred at similar, but slightly elevated, pressures, while in hydrogen only slight paralysis was observed at 200 atm. The protection afforded against the onset of paralysis by these gases and other anesthetics (14) has not been explained. In general then helium and neon exert effects comparable to hydrostatic pressure and neither gas appears to give rise to general anesthesia in the range of mechanically tolerable pressures,

[1] From Session II, **Physiological Responses to the Environment**, of the FASEB Conference on *Organismal Response to the Environment* presented at the 60th Annual Meeting of the Federation of American Societies for Experimental Biology, Anaheim, CA, April 13, 1976.

[2] Pressures are given in standard atmospheres (1.03 $kg \cdot cm^{-2}$). 10 meters depth of sea water is roughly equivalent to one atmosphere.

HPNS is high pressure neurological syndrome.

although the next rare gas, argon, is an anesthetic at 20 atm partial pressure.

The physicochemical changes produced in these pressure ranges are in general not great. Equilibriums and reaction rates will be affected by about a factor of 2 or so at 100 atm, but by 1 to 2 orders of magnitude at the ocean's deepest depths (~1,100 atm), processes involving a volume increase being opposed by pressure (16). One might predict then that in the physiological pressure range (say <300 atm) only those processes in which greater than normal volume changes occur, or in which fairly critical temporal integration takes place, would be influenced significantly by pressure. A recent example of the latter type may be the pressure-induced bradycardia observed in isolated mouse sinus nodes (21). The spontaneous contraction of muscle is associated with an unusually high volume change of 350 ml/mole (6), which may be related to the observation of high pressure paralysis.

Detailed studies with mammals have been carried out over a smaller pressure range than those with aquatic animals. Although pressures as high as 300 atm have been obtained briefly, the animals are generally in poor condition above 200 atm even when the other environmental stresses, such as temperature, are controlled. When mice are compressed in an $He-O_2$ atmosphere a series of responses are observed. First, an uncoordinated trembling of the limbs and jerky voluntary movements are observed with onset pressures of 25–60 atm, depending on the compression rate. Second, convulsions, both clonic and clonic-tonic, occur at higher pressures than do the tremors. The threshold pressure for onset of convulsions may be elevated by adding an anesthetic gas to the $He-O_2$ mixture. Mouth breathing is observed at about 90 atm, but this appears to be unrelated to gas density since it occurs at the same pressure in neon–oxygen mixtures which are 5 times as dense. Finally the mice die at about 140 atm, but this event may be postponed by using slower compression rates or, more effectively, by adding anesthetic gases to the breathing mixture (3, 14). Several elegant studies, in which mice totally immersed in a highly oxygenated fluorocarbon liquid were compressed hydraulically, have demonstrated that these effects are mediated by pressure per se and are not to be attributed to helium (13).

The use of anesthetic gases to ameliorate the effects of pressure reintroduces the problem of inert gas narcosis into deep diving. However this problem is mitigated by the remarkable observation that pressure reverses the effects of anesthetics (9, 10, 14). This raises the possibility that the high pressure neurological syndrome may be controlled using trimix (e.g., He, N_2, O_2) without incurring inert gas narcosis (19). The symmetry of the situation is summarized in Table 1, where data for the elevation of convulsion threshold by addition of nitrogen and for the depression of anesthetic potency by addition of helium are presented. At present the most successful approach for assessing the role of inert gases in modifying the effects of pressure per se is the approach based on simple physicochemical concepts developed to explain the mechanism of action of anesthetic agents. These concepts enable quantitative predictions to be made of the optimum balance in the trade-off between inert gas narcosis and the HPNS. They also provide a conceptual link between the molecular level interaction of the anesthetics with their site of action in a membrane and the behavior of the whole animal. Thus anesthetic potency correlates remarkably well with lipid solubility. However the pressure reversal of anesthesia suggests that the anesthetics not only dissolve in, but also expand and fluidize, the lipid bilayer regions of biomembranes (10, 20). Anesthetics have in fact been observed to fluidize membranes (17, 23) and this fluidization is reversed by pressures of the same magnitude as those observed physiologically (23). Anesthetic-induced membrane expansion has also been measured and found to be consistent with that required for anesthesia (22). If an increase of membrane volume or fluidity can lead to anesthesia, then it seems probable that a corresponding decrease may also result in marked changes in membrane function such as the excitability associated with the high pressure neurological syndrome (HPNS). The mechanism by which these changes in fluidity of the lipid bilayer could give rise to such profound events as anesthesia and the HPNS remains to be clarified. Certainly the changes induced in the lipid bilayers themselves are small, but the emerging relationship between lipid fluidity and the function of membrane protein suggests plausible mechanisms. Electrophysiological studies may provide further insight (4, 7, 11).

TABLE 1. Partial pressure of nitrogen to cause anesthesia or prevent hyperbaric convulsions in mice at various pressures

Total pressure	Nitrogen partial pressure at	
	Anesthesia	Convulsions
39	38	—
69	44	0
93	46	9.4
110	48	17
119	49	32

Pressures in atmospheres. Anesthesia data (Wilson and Miller, unpublished). Convulsion data ref 3. Gas mixtures contain 1 atm O_2. The balance is helium.

We shall now consider the formulation, testing, and application of the critical volume hypothesis, which may be stated in two forms. First, anesthesia occurs when the volume of a hydrophobic region is caused to expand beyond a certain critical volume by absorption of an inert substance. An applied pressure opposes this expansion and reverses the anesthesia. Second, convulsions occur when some hydrophobic region has been compressed beyond a certain critical amount by the application of pressure. Absorption of an inert gas will compensate for such compression and raise the convulsion threshold pressure.

These hypotheses can be tested against data of the type given in Table 1. To calculate the expansion caused by an anesthetic when it dissolves in a hydrophobic fluid it is necessary to know the solubility in the membrane involved. Unfortunately few such data are available and in any event the exact site of action, and hence the appropriate membrane composition, is unknown. Because of this a number of model solvents have been used; olive oil, benzene, and carbon disulfide provide particularly good analogs and the conclusions derived turn out not to be particularly solvent dependent. It is also possible to test the hypotheses algebraically without assuming a model solvent (20). This becomes very cum-

bersome however, especially at higher pressures where a number of corrections for nonideality must be made. In this paper the calculations are illustrated using benzene as the model because the required physical parameters have been most accurately determined for this solvent.

The required equations are set down in Fig. 1. The gross fractional expansion caused by dissolving a gas is calculated from a knowledge of the partial molar volume of the gas in the solvent. The mechanical compression resulting from elevating the partial pressure is given by the compressibility. Two corrections need to be taken into account: one for deviation of the gas mixture from ideality and the other resulting from the reduction in solubility of the gases at high pressure (deviations from Henry's law) (20). With typical diving gases these corrections reduce the calculated expansion by 10–20% at 100 atm. (This is not sufficient in itself to account for pressure reversal.) In a dissolved gas mixture the total gross expansion is given by the sum of the individual terms. For a given physiological end point, which may be achieved at different pressures according to the gas mixture employed (e.g., Table 1), the total gross expansion may be calculated. Plotting this versus the total pressure should yield a straight line whose slope is the compressibility and whose intercept is the critical volume change as illustrated in Fig. 2. In Fig. 3 data for iso-anesthetic and iso-convulsive end points are tested in this way. The fit is acceptable, a volume change of around 1% leads to anesthesia (expansion) or convulsions (compression), and the site of action of convulsions is 4–5 times as compressible as the anesthetic site (18). The degree of agreement is remarkable for such a simple model.

Although a number of uncertainties surround the conclusions these are probably not serious. Thus the convulsion threshold depends on the strain of mice—an A/J strain in this case which convulses at a lower pressure than the CD strain used for the anesthesia work. The limited data on convulsions in CD mice suggest they exhibit a lower compressibility and a lower critical volume than A/J mice (K. W. Miller, unpublished calculations). In either case, however, the site of action of convulsions is distinct from that for anesthesia. Furthermore no model as simplistic as the critical volume hypothesis can be expected to account for the neurological accommodation or adaptation implied by the compression rate dependence of the convulsion threshold. However the data used here were all obtained with a uniform compression rate of 40 atm/hr and the convulsion thresholds differ from this by no more than 20% at higher and lower rates.

Another shortcoming of the model follows from the conclusion that there are distinct sites of action mediating the anesthetic and convulsant sites. This conclusion can also be reached algebraically without assuming a particular solvent model (K. W. Miller, unpublished calculations). However, in the model calculations the gas solubilities of these two sites have been assumed to be identical so that the difference must perforce appear entirely in the compressibilities. These problems reflect our ignorance. No black-box approach such as this can be expected to do more than provide a self-consistent description and verify in a general way the underlying concepts. The high degree of consistency of the model, however, is challenging and points the way to more detailed biophysical investigations which alone can provide a direct test of the underlying assumptions.

The critical volume hypothesis can now be used to predict the composition of gas mixtures required to avoid both anesthesia and convulsions. It may be seen immediately that no single mixture can prevent a volume change at both sites, and this is bound to impose a limiting pressure beyond which both end points cannot be avoided simultane-

Figure 1. The net expansion, E, occurring when a gas dissolves in a fluid is given in the upper equation for the second component of a gas mixture. $\bar{V}$ is the partial molar volume of the gas dissolved in the fluid of molar volume V_m; x is its mole fraction solubility at a partial pressure of 1 atm, and P is the partial pressure of the gas. β is the compressibility of the solvent. Two correction terms must be applied to the first term on the right, and these are given. P^* is the fugacity of a gas at partial pressure P_1 in a mixture of two gases, X_1 and X_2 are the mole fractions of the gases in the mixture, and B the second virial coefficient. The second correction is for deviations from Henry's law. C is the mole fraction concentration at partial pressure P. P_T is the total pressure, R the gas constant and T the absolute temperature. For further details see (20).

CRITICAL VOLUME HYPOTHESIS

Net Expansion – each gas

$$E_2 = \frac{\bar{V}_2 \cdot x_2 \cdot P_2}{V_m} - \beta \cdot P_2$$

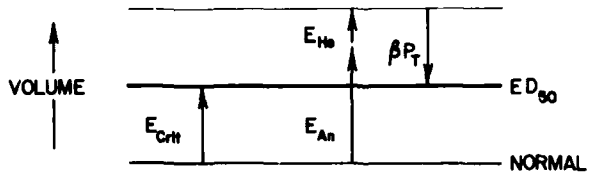
Correction terms

(1) Gas imperfections

$$\ln\left(\frac{P^*}{P_1}\right) = \frac{1}{RT}\int_0^{P_T}\left[x_1 \cdot B_{11} + x_2 \cdot B_{12}\right] dP$$

(2) Dependence of solubility on pressure

$$\frac{d\,(\ln(c/P))}{dP_T} = -\frac{\bar{V}_2}{RT}$$



$$E_{Crit} = (E_{He} + E_{An}) - \beta P_T$$

$$(E_{He} + E_{An}) = \beta P_T + E_{Crit}$$

Figure 2. Equations for testing the critical volume hypothesis for anesthetic action. E_{crit} is the critical expansion which results in anesthesia in half of a group of animals (ED_{50}). E_{An} is the expansion caused by the dissolved anesthetic at a concentration greater than its ED_{50}. E_{He} is the expansion caused by helium dissolved at a pressure that reverses anesthesia. β is the compressibility and P_T the total mechanical pressure.

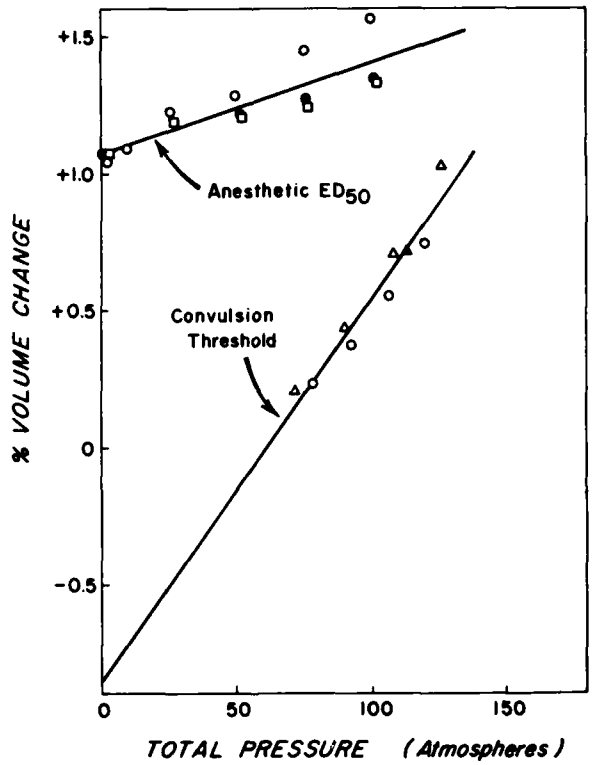


Figure 3. The percent volume change calculated for the gas mixtures (e.g., $E_{He} + E_{An}$) that give rise to an anesthetic ED_{50} or a convulsion at the total pressure P_T. ○ $He-N_2O$; △ $He-N_2$; □ $Ne-N_2O$; ● H_2N_2O; ▲ H_2 alone. For data sources see (18). Reproduced with permission from reference (18). Copyright 1974 by the American Association for the Advancement of Science.

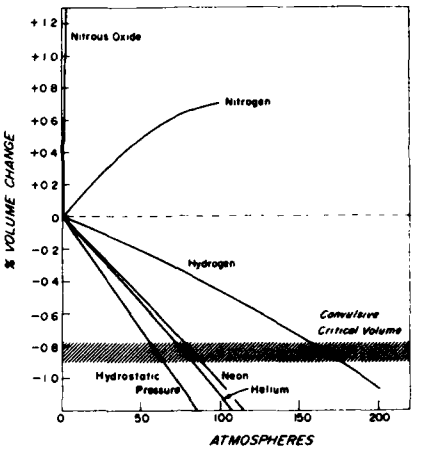


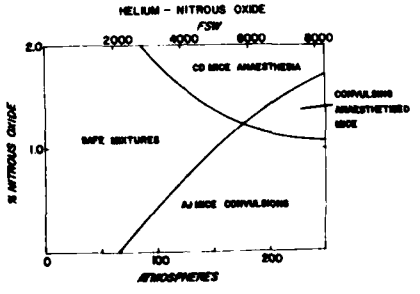
Figure 4. The net volume change (e.g., $E_{He} - \beta P_T$) calculated for the site of action of convulsions using the compressibility and critical volume derived from Fig. 3. The shaded area indicates the critical contraction at which convulsions first occur.

ously. In addition since anesthetics and pressure are both nonspecific in action it seems probable that there is a spectrum of similar sites some of which might be involved with control of vital cardiac and ventilatory processes. The roles of these in limiting the physiologically tolerable pressures are unknown. Furthermore there are unrelated mechanisms where pressure or anesthetics may act either independently of each other or, more seriously, even synergistically. Such an effect has been reported, for example, on the slowing of the rate of beating of the pleopods of *Marinogammarus marinus* (12) by 0.005 atm of halothane ($CF_3CHClBr$), which is enhanced by application of 136 atm, a pressure which in the absence of anesthetic has no effect.

In spite of these possible limitations the mixtures predicted by the critical volume hypothesis appear realistic in so far as they have been tested. Thus if one calculates the net volume change that occurs when compression is carried out hydraulically, or with a number of inert gases, the correct ranking order of convulsion thresholds is obtained. Nitrogen is found not to cause convulsions because its solubility is high enough to result in net expansion, while with helium and neon net contraction occurs mainly because of their low solubility (Fig. 4). The result of mixing a strongly expanding gas, such as N_2O, with helium is to reduce the net compression. The mixtures corresponding to expansion sufficient to cause anesthesia or compression sufficient to cause convulsions are summarized in Fig. 5. Here the optimum mixture for the point where the anesthesia and convulsion thresholds converge is seen to contain a little over 1% N_2O. Beyond this pressure it is necessary to provide deeper and deeper anesthesia to prevent convulsions. We have recently confirmed the latter prediction in our laboratory. With CD mice it is possible to prevent convulsions elicited by sound without loss of righting reflex at 130 atm total pressure, but at 200 atm complete loss of righting reflexes occurs before these convulsions can be prevented. One thus achieves the state of a convulsing anesthetized mouse, which confirms the prediction that the two sites of action are indeed separate! Because of the phenomenon of accommodation to pressure, Fig. 4 does not define the ultimate pressure limits. However even with cautious compression schedules the condition of mice at pressures above 200 atm is rarely good. Further extension of pressure tolerance cannot then be achieved with gas mixtures. We do find that agents such as phenobarbital have some advantages, but more specific pharmacological intervention will be required to significantly raise the tolerable threshold further. Whether it will be possible to breed animals with tolerance to pressure remains to be seen.

It is possible to extend this analysis to include predictions for manned

Figure 5. The total pressure at which anesthesia or convulsions will be observed calculated as a function of percentage of nitrous oxide mixed with helium. FSW, feet of sea water.



diving. Here the acceptable gas mixtures are bounded by the inert gas narcosis and tremor thresholds. These are considerably more subjective phenomenons than convulsions and general anesthesia and consequently there are less reliable objective data available. Sufficient accounts of nitrogen narcosis in mixtures of helium and nitrogen have been given to allow one to calculate that the compressibility at this site of action does not differ significantly from that for anesthesia in mice, although of course the critical volume is much smaller. It is thus possible to estimate that a mixture of 10% nitrogen and 90% helium should not cause significant narcosis at any pressure (K. W. Miller and M. Wilson, unpublished calculations). How far the percentage of nitrogen needs to be raised to avoid significant hyperexcitability is not yet clear; nor are any systematic data available for testing the theory. However, the use of 5–10% nitrogen in helium does appear to improve performance following rapid compression to depths of about 300 m (2). Ft

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Anesthesiology
48:104-110, 1978

Reprinted from: ANESTHESIOLOGY, Vol. 48, No. 2, February 1978

The Pressure Reversal of a Variety of Anesthetic Agents in Mice

Keith W. Miller, D.Phil., and Michael W. Wilson, B.S.†*

The aim of this work was to study in mammals the ability of high pressures to reverse the anesthesia produced by a wide range of general anesthetics. Dose–response curves were obtained using mice at pressures ranging from 1 to 125 atm for five agents, namely α-chloralose, ethylcarbamate, phenobarbital and, for comparison, nitrogen and argon. The increase of ED_{50} was found to be a linear function of pressure in each case, but the proportionate increases in ED_{50} with pressure were greater for the three non-inhalation agents than for the two gases. Thus, the ratio of ED_{50} at 100 atm to that at 1 atm was 1.74 for α-chloralose, 1.68 for ethylcarbamate, and 1.54 for phenobarbital. On the other hand, the corresponding ratios for argon and nitrogen were only 1.36 and 1.34. The potencies of three short-acting agents (trichloroethanol, ketamine, and alphadione) were shown to increase with decreasing pressure, although ED_{50} values could not be obtained. It is concluded that pressure reverses the actions of a wide variety of anesthetics in mice. The results of this study are not inconsistent with either the fluidized lipid membrane or the critical volume hypotheses of anesthetic action. (Key words: Theories of anesthesia, critical volume; Hyperbaria, reversal of anesthesia; Anesthetics, intravenous, ketamine; Anesthetics, intravenous, steroid, alphadione; Hypnotics, barbiturates, phenobarbital; Hypnotics, α-chloralose; Hypnotics, urethane.)

IN THE FIRST DEMONSTRATION of the pressure reversal of anesthesia, Johnson and Flager (1950) showed that anesthesia induced in tadpoles by ethanol and urethane was abolished by increasing the pressure.[1] Subsequent studies of inhalation agents in newts and mice have lent support to the concept that anesthetics act by expanding hydrophobic regions in the central nervous system (the critical volume hypothesis).[2–4] Few studies of intravenous agents in mammals have been reported, however, largely because the ambiguities that are imposed by pharmacokinetics restrict such studies to favorable cases[5] or to technically difficult procedures.[6] In this study, we examined the interactions between pressure and anesthetic potencies in mice of six intravenous agents, and, for comparison, two gaseous agents. We were able to demonstrate unequivocally pressure reversal even with moderately short-acting agents. In each case we confirmed our result by demonstrating that pressure reversal was reversible, as follows: after anesthesia had been reversed by applying pressure it was reimposed by lowering the pressure again, thus showing that the hyperbaric, rather than the temporal, vector was responsible for the reversal of anesthesia. Our data suggest that the concepts of the critical volume hypothesis may be applied to a wide range of general anesthetics acting in mammals.

Methods

Two types of pressure chamber were employed. A single 34-liter chamber was used for the longer-acting agents, whereas three 300-ml chambers allowed the more rapid changes in pressure required for the shorter-acting agents. The 34-liter chamber is constructed from a flanged cruciform cast-iron steam pipe with a 7-inch ID, fitted at each end with a window fashioned from 4-inch-thick plexiglass held in aluminium retainers sealed with "O" rings. This chamber accommodates two seven-compartment rotatable, cylindrical cages placed on carriages in front of the windows. Two mice, probed for rectal temperature, are placed in the center of the chamber. A muffin fan in the third arm and a heat exchange unit with fan in the fourth arm provide efficient gas mixing and heat distribution. Set temperature is maintained ± 0.1 degree C by circulating water through the internal heat exchanger and an outer jacket. The temperature is controlled by a thermistor in the chamber, which operates a valve allowing short pulses of either hot or cold water into the heat exchanger.

The smaller stainless steel chambers are those described earlier.[7] One is fitted with a thermistor, and they are heated by an external hot air blower. A temperature increase of 3 C resulted from a typical compression.

Pressure in the large chamber is monitored by one or more of three Heise‡ bourdon-tube gauges. Pressure in the smaller chamber is measured with a Marsh§ Master Test 200 gauge, measuring to 5,000 psi.

Metabolic gases were periodically checked by gas chromatography. They were controlled by soda lime in the small chambers and a soda lime–silica gel-activated charcoal (3:3:2) sandwich in the large chamber. Water is produced both by the soda lime and by

* Associate Professor of Pharmacology in the Department of Anaesthesia, Harvard Medical School.

† Research Assistant, Massachusetts General Hospital.

Received from Harvard Medical School, Departments of Anaesthesia and Pharmacology, Massachusetts General Hospital, Boston, Massachusetts 02114. Accepted for publication July 20, 1977. Supported by a contract from the Office of Naval Research, Physiological Branch (Contract N00014-75-C-0727), with funds provided by the Naval Research and Development Command. K. W. Miller is supported by a Public Health Service Research Career Development Award (GM 00199) from the National Institute of General Medical Sciences.

Address reprint requests to Dr. K.W. Miller.

‡ Newington, Conn.

§ Wilmette, Ill.

0003-3022-78-0200—0104$00.85

the mice, and we found that the silica gel controlled chamber humidity sufficiently to prevent the windows from fogging. It and the charcoal also controlled ammonia and other odors.

Response to anesthetics was measured as before.[7] The cages (or small chambers) were rotated at 4 revolutions/min and the ability of the mouse to remain upright observed in each of five complete revolutions (the rolling response). A score between zero and five could thus be obtained. Scores were recorded as percentages.

All experiments were carried out using male CD-1 mice (Charles River) weighing 20–30 g. Anesthetics were administered intraperitoneally. All doses were adjusted in proportion to body weight. Ethylcarbamate or urethane (Fisher), 2,2,2-trichloroethanol (Fisher), and ketamine hydrochloride (Ketalar, Parke Davis) were dissolved in physiologic saline solution before injection; α-chloralose (Aldrich) was dissolved in saline solution containing ethylene glycol, 40 per cent, before injection. Phenobarbital was supplied in solution (Invenex Pharmaceuticals) and diluted in saline solution. Alphadione (Althesin) was a gift of Dr. G. H. Philips (Glaxo, U.K.) and contained alphaxalone and alphadolone acetate (3:1) dissolved in saline solution containing 20 per cent polyoxyethylated caster oil.

After injection, the mice in their cages were sealed into the large chamber, which was flushed with oxygen for 5 min. The average time between injection and compression with helium was about 30 min. The rate of pressurization was kept at about 2 atm/min, and chamber temperature was maintained at about 35 C in order to maintain rectal temperature at 37 ± 1 C. The response of the animals was measured with increasing pressure and time until it was 100 per cent. Decompression was then begun, usually at about 1 atm/min, and the response measured as before.

For gaseous anesthetics the inert gas partial pressure was increased to the desired value after oxygen flushing was completed. Successive doses of gas could be added until a complete dose–response curve had been defined. The pressure was further increased with helium until pressure reversal occurred, and then another anesthetic dose–response curve was obtained. Compression rates were much slower (50 atm/hr) than with the other anesthetics because the same mice were also used later in experiments for observations of the effects of extremely high pressures (~200 atm), which will be reported elsewhere. The animals were thus partially pressurized late on the first day and the dose–response curves obtained the following morning.

With the shorter-acting anesthetics each mouse after injection was sealed in a small chamber, 0.5 atm oxygen added, the response measured, and the chamber then pressurized with helium. The time between final injection and pressurization could be as little as 3 min. Temperature regulation was observed to be less critical in the short exposures in these small chambers. No immediate change in response was elicited by rapid changes of temperature in the range 27–36 C. After compression (at about 8 atm/min) to the maximum pressure of the experiment, animals were observed until recovery of the rolling response occurred; then the pressure was rapidly decreased by as much as 60 per cent and the rolling response observed. In some experiments the pressure was increased and decreased several times. Controls showed no signs of decompression sickness[8] during such procedures.

Dose–response curves were analyzed on a digital calculator using the method of Waud[9] for quantal responses. The scale parameter (table 1) provides a measure of the slope of the dose–response curve.

All pressures are absolute unless specifically stated to be gauge.

TABLE 1. Variation of ED_{50} for Loss of Rolling Responses with Total Pressure

Agent	Total Pressure ATA	ED_{50} ± SE g/kg	Number of Animals	Scale Parameter* ± SE
Phenobarbital	1	.113 ± .0040	63	−12 ± 2.8
	2	.112 ± .0036	42	−14 ± 4.6
	50	.147 ± .0074	28	−15 ± 5.9
	75	.162 ± .0045	21	−23 ± 6.9
	100	.174 ± .0042	28	−24 ± 8.9
	75*	.15 ± .021	14	−7.6 ± 7.7
α-Chloralose	2	.0301 ± .00092	34	−13 ± 5.9
	10	.0314 ± .00095	70	−13 ± 3.1
	50	.0405 ± .00081	49	−21 ± 7.0
	25*	.0366 ± .00067	28	−27 ± 9.6
Ethyl carbamate	1	.97 ± .051	14	−17 ± 8.5
	2	1.01 ± .051	14	−19 ± 9.3
	40	1.32 ± .021	35	−55 ± 26
	70	1.45 ± .061	28	−24 ± 8.4
	52*	1.30 ± .055	24	−13 ± 6.5
Argon	19.1	18.1 ± .67 atm	35	−11 ± 4.6
	81	21.4 ± .78	17	−14 ± 8.7
	124	24.2 ± .59	14	−32 ± 16
Nitrogen	39.9	38.9 ± .94 atm	63	−12 ± 3.8
	81	45.7 ± .66	28	−32 ± 14
	121	48.8 ± .50	28	−46 ± 20

* Values obtained on decompression.

Results

Phenobarbital provided sleep times in excess of eight hours. Responses were measured only between three and six hours after injection. Analysis of the dose–response curves from a number of experiments

shows that ED_{50} increases with pressure (table 1). Furthermore, an ED_{50} of 0.16 ± 0.005 g/kg at 75 atm during compression compares well with one of 0.15 ± 0.02 g/kg later during decompression from 100 atm. In a few experiments we obtained a similar result on decompression to 50 atm. Thus, a response of eight of nine mice at 100 atm because zero of nine on lowering the pressure to 50 atm ($P \ll 0.005$).

Ethylcarbamate (1.2 g/kg) provided a sleep time of four hours. Dose–response curves were obtained at 1, 2, 40, and 70 atm. The ED_{50} increased with pressure from 1.0 g/kg at 1 atm to 1.45 g/kg at 70 atm (table 1), and the ED_{50} obtained on lowering the pressure was consistent with that obtained earlier during compression. In one experiment a response of nine of ten mice at 70 atm became four of ten at 52 atm ($P = 0.03$).

Sleep times with α-chloralose were little more than an hour, but induction took 30–60 minutes, so it was possible to load the large chamber and obtain complete dose–response curves at pressure. However, several additional problems were encountered with this agent. First, dose-dependent uncoordinated activity and twitching were a feature of α-chloralose anesthesia that appeared to be exacerbated by pressure. By way of contrast, the other agents tested gave good protection against the hyperexcitability normally encountered at pressure.[10] However, with α-chloralose above about 65 atm, extremely uncoordinated movements, and even mild clonic convulsions, prevented us from obtaining meaningful righting reflex data at still higher pressures. Consequently, all work was carried out at or below 50 atm. Second, rectal temperatures of animals injected with α-chloralose were extremely sensitive to environmental temperature, increasing, for example, 0.8–1.5 C during compression from 10 to 50 atm and then rapidly returning to their initial value. Furthermore, the observed response was unusually sensitive to rectal temperature. Consequently, rectal temperature was closely controlled in the range 36.7–37.2 C. Even so, an additional variable was found to be the presence of helium. Thus, at 1 atm oxygen plus 1 atm helium an ED_{50} of 30 mg/kg was determined, while in air in the chamber 70 per cent of animals righted at 36 mg/kg, even though the rectal temperature was slightly lower than usual. This effect of helium was not further investigated, but we carried out all measurements in the presence of at least 1 atm helium and included an extra determination at 10 atm. The ED_{50} values of phenobarbital and ethylcarbamate were found to be the same in 1 atm oxygen and 1 atm oxygen plus 1 atm helium (table 1). With these precautions, the dependence of ED_{50} on pressure was found to be essentially linear and of about the same magnitude as for phenobarbital and for ethylcarbamate (table 1). An ED_{50} obtained on decompression from 50 to 25 atm confirmed the reversibility of pressure reversal. A response of six of seven mice at 50 atm decreased to one of seven at 25 atm ($P = 0.002$) in one experiment.

Mice anesthetized with trichloroethanol had rather variable sleep times; nonetheless, marked and reversible effects of pressure were individually demonstrated. Thus, three mice received 311.4 mg/kg. The first awoke 40 min later at 136 atm. The pressure was decreased in 5 min to 54 atm, reanesthetizing the mouse for a further 5 min. The second awoke 30 min later at 116 atm and was reanesthetized for a further 30 min by lowering the pressure to 48 atm. The rolling response was again restored for a few minutes by increasing pressure to 116 atm, lost on decreasing pressure to 68 atm, and after 8 min restored to an average of 50 per cent by increasing pressure to 85 atm. The third mouse, after 27 min at 119 atm, had an average rolling response of 50 per cent, which was increased to 100 per cent at 129 atm and reduced to 20 per cent at 122 atm. A fourth mouse received 233.6 mg/kg and awoke after 6 min at 34 atm, was anesthetized again by lowering the pressure to 13 atm, and reawoke on increasing the pressure to 34 atm.

Alphadione (40 mg/kg) sleep times ranged from half an hour to an hour. Two mice were pressurized in small chambers to 130 atm. After recovery of rolling responses (21 and 25 min after injection), the pressure was decreased to 55 atm, completely restoring anesthesia. Rolling responses at this pressure were recovered in both cases 35 min after injection. Longer sleep times were produced by repeated injection. Six mice received injections of 100 mg/kg, with two to four subsequent doses of 50 mg/kg on awakening, and were then pressurized in small chambers to 90 atm. Rolling response was regained after periods ranging from 20 to 105 min. As before, pressure was decreased in each case at this point, yielding five mice with complete loss of rolling response (one at 62 atm and four at 35 atm). The sixth mouse had an average rolling response of 50 per cent at 35 atm. All animals recovered from anesthesia at the lower pressure within 60–165 min after the final injection.

The sleep time with ketamine, even after repeated injections, rarely exceeded 30 min. This made unequivocal demonstration of pressure reversal difficult. In three mice given 100 mg/kg the rolling response recovered at 68 atm after 15 to 30 min. Anesthesia was restored for more than 5 min by lowering the

pressure to 20 atm. A fourth mouse, which recovered the rolling response after 10 min at 68 atm, was reanesthetized for 5 min by decreasing pressure to 44 atm. All four animals regained normal behavior at pressure. In another case anesthesia was not restored by lowering pressure to 20 atm, which was the practical lower limit of pressure imposed by the oxygen levels.

Because of the uncertainties surrounding the results with ketamine and alphadione we confirmed them with experiments on 10–12-day-old rats, which have sleeping times of 2–4 hours in the large chamber. In one experiment a dozen 12-day-old rats were given 30 mg/kg ketamine intraperitoneally. Their response 55 min after injection was 50 per cent at 100 atm. They were decompressed at 12 atm/min to 50 atm; after 10 min their response was 0 per cent ($P = 0.007$). They were then recompressed at 12 atm/min to 100 atm, where they gave a response of 67 per cent. Similarly, with alphadione 40 mg/kg gave three and a half- to four-hour sleep times. Nine rats had responses of 67 per cent at 100 atm 85 min after injection, zero at 50 atm 98 min after injection ($P < 0.005$), and 100 per cent at 100 atm 104 min after injection. Nine other rats had responses of 72 per cent at 100 atm 90 min after injection, zero at 50 atm 104 min after injection ($P < 0.005$), and 55 per cent at 50 atm 164 min after injection.

One general observation from the quantitative studies was the tendency for the dose–response curves to become steeper with pressure (*i.e.*, the scale parameter[9] becomes more negative). This had been noted previously in the phenobarbital study.[5] Our data cover four more anesthetics. Regressing the 21 unweighted scale parameters in table 1 against pressure yields a slope of -0.45 ± 0.15, an intercept of -6.5 ± 10.1, and a correlation coefficient of 0.55. The F test concludes this slope to be less than zero with 99 per cent confidence. Thus, the population homogeneity of our sample of animals apparently increases with pressure. Intuitively, one would have expected pressurization to increase the scatter in our data because of the introduction of additional variables. One possible explanation is that the heat losses imposed by hyperbaric helium, together with our close control of environmental temperature, may actually have imposed a more uniform temperature on the group of mice.

Discussion

We were able to demonstrate pressure reversal with all the anesthetics we examined. This conclusion is supported by other studies, some of them only semiquantitative and carried out in tadpoles. Thus, our data for nitrogen and argon are broadly consistent with similar data of R.A. Smith, *et al.*,¶ obtained in mice, except in one particular. These investigators claim that the ED_{50} of these gases is not a linear function of pressure. We have analyzed their data and find that the curvilinearity is significant for nitrogen only (comparing a linear with a quadratic regression by analysis of variance, the F-test yields for argon $P > 0.05$ and for nitrogen $P < 0.01$). Our own less extensive data do not confirm this nonlinearity, nor does previous work with newts,[3] but more detailed work is needed to resolve this point. Our data for phenobarbital agree well with results of a previous study at 103 atm[5] by the same investigators. In addition, our study shows the ED_{50} to increase linearly and reversibly with pressure. Thus, our data for three anesthetics are in most respects in satisfactory agreement with independent work. Our quantitative data for α-chloralose and ethylcarbamate show relations between ED_{50} and pressure similar to that of phenobarbital. This is the first demonstration of pressure reversal with α-chloralose; an early qualitative study with tadpoles confirms our ethylcarbamate data.[1] Although α-chloralose pressure-reversed normally, it differed from the other anesthetics in not protecting against high-pressure excitability and in its sensitivity to low partial pressures of helium. Our work provides no explanation for these effects, but others have reported effects of helium at low partial pressures on the sympathetic nervous system and cardiovascular function.[11] Our demonstration of reversible pressure reversal of trichloroethanol, alphadione and ketamine in mice and young rats is new. The results are consistent with a decreased ketamine sleep time observed in guinea pigs at pressure[6] and the reversal of alphadione anesthesia in tadpoles.[12] More quantitative work than this in mammals will require determination of drug levels *in vivo*.

Our data extend to mammals the conclusion that the potencies of a wide range of anesthetic agents may be decreased by increased pressures.[12] Although these agents differ in many of their effects, it has often been supposed that they produce anesthesia by a common mechanism.[12–14] That these nonspecific agents are antagonized by a nonspecific agent such as pressure is appropriate, and is consistent with the above-mentioned view. Presumably, the anesthetic–

¶ Smith RA, Winter PM, Halsey MJ, et al: Helium pressure produces a non-linear antagonism of argon or nitrogen anesthesia in mice. Abstracts, American Society of Anesthesiologists, 1975, pp 217–218.

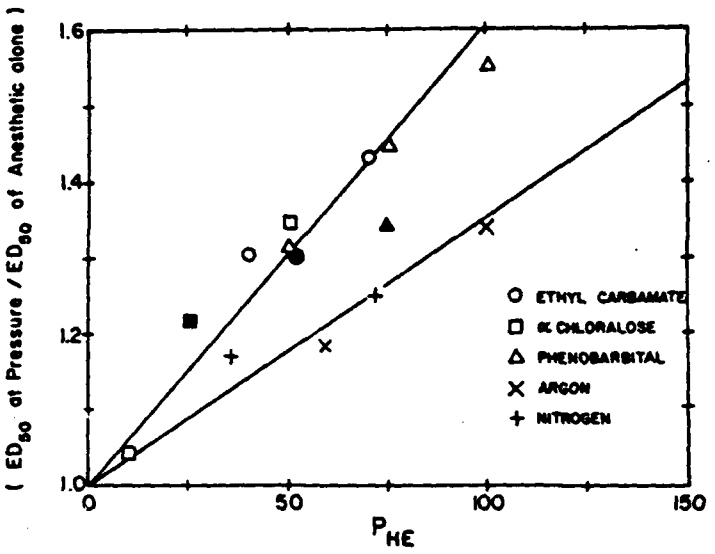

FIG. 1. Results for non-inhalation anesthetics plotted according to equation 4 and for gaseous anesthetics according to equation 5. The units of P_{He} are atmospheres. The lines were fitted by least-squares analysis through the origin. Since the standard deviations of the ED_{50}'s (table 1) vary considerably, each point was weighted by the reciprocal of the sum of the variances of the ED_{50} of the anesthetic alone and at pressure. The sum of the weights has been normalized to equal the number of data points. The slope (±standard deviation) for the non-gaseous agents is $6.1 \times 10^{-3} \pm 0.24 \times 10^{-3}$ and that for the two gases $3.5 \times 10^{-3} \pm 0.19 \times 10^{-3}$. The solid symbols represent ED_{50} values obtained during decompression.

pressure interaction must occur at several sites, not all of which are involved in anesthesia. In fact, pressure studies have distinguished one site where pressure reverses anesthesia from another where anesthetics reverse high pressure-induced convulsions,[15,16] but in general more detailed studies will be needed to resolve such questions. One such study shows that the nerve block occasioned by charged local anesthetics is not pressure-reversed, while that of the uncharged local anesthetics is.[17] This is consistent with the view that while the latter form acts nonspecifically, the charged form acts at a specific site and cannot be reversed by pressure.[18] In this case pressure provides a tool for distinguishing the two actions, as it may do in less well understood situations.[19]

Our results may be examined in the light of two current views of the mechanism of general anesthesia. These are the so-called critical volume hypothesis and the fluidized lipid hypothesis.

General anesthetics fluidize lipid bilayer membranes containing cholesterol, whereas lipid-soluble non-anesthetics (*e.g.*, tetradecanol) do not, and partial anesthetics (*e.g.*, tetrahydrocannabinol) do so to only a limited extent.[20–24] This effect is reversed by pressure.[20,21] Some of the agents we studied have already been reported to fluidize membranes.[22–24] In addition, α-chloralose, phenobarbital and trichloroethanol fluidize such lipid bilayers (Pang and Miller, unpublished observations). Thus, our data are self-consistent with the fluidized lipid hypothesis when cholesterol: phospholipid bilayers are used as a model of the site of action.

We may now use our quantitative data for intravenous anesthetics to test the critical volume hypothesis, which previous tests have confirmed for gaseous anesthetics in newts[3] and mice.[15] First, we extend the previous derivation for gases to non-inhalation agents, for which the fractional expansion may be written:

$$E^1_{50} = \frac{C^1_{50}\bar{V}_a}{V_m} \qquad (1)$$

where E^1_{50} is the fractional expansion of the anesthetic site of action at 1 atmosphere (superscript) when an ED_{50} concentration of anesthetic, C^1_{50}, is achieved. $\bar{V}_a$ is the partial molar volume of the anesthetic and V_m the molar volume of the site of action. The fractional expansion, E_{He}, caused by increasing the partial pressure of helium, P_{He}, is given by[3]:

$$E_{He} = \left(\frac{P_{He}x_{He}\bar{V}_{He}}{V_m}\right) - \beta P_{He} \qquad (2)$$

where x_{He} is the mole fraction solubility and $\bar{V}_{He}$ the partial molar volume of helium at the anesthetic site of compressibility β. For all simple solvents it is found that the compressibility term in equation 2 is larger than the expansion term due to helium dissolving, so net compression results. For more soluble gases (larger x), net expansion occurs. If C^P_{50} is the concentration at the anesthetic site required to produce an ED_{50} response at total pressure P_T, then according to the critical volume hypothesis:

$$E^1_{50} = \frac{C^1_{50}\bar{V}_a}{V_m} = \frac{C^P_{50}\bar{V}_a}{V_m} + E_{He} \qquad (3)$$

whence:

$$\frac{C^g_{50}}{C^l_{50}} = \left(\frac{\beta}{E^l_{50}} - \frac{\bar{V}_{He}x_{He}}{E^l_{50}V_m}\right)P_{He} + 1 = AP_{He} + 1 \quad (4)$$

Similarly, for gaseous anesthetics it has been shown that[3]:

$$\frac{P^g_{50}}{P^l_{50}} = A(P - P^g_{50}) + 1 = AP_{He} + 1 \quad (5)$$

where P^l_{50} is the ED_{50} partial pressure of the pure gas, P^g_{50} is the ED_{50} partial pressure in the presence of additional helium pressure, and P is the total pressure. If the sites and mechanisms of action of gaseous and non-gaseous anesthetics are identical, then linear plots of equations 4 and 5 should have equal slopes. Before such a test may be made, however, equation 4 needs to be modified to experimental variables by the assumption that the ratio of total doses per unit weight are an adequate representation of the ratio (C^g_{50}/C^l_{50}).

Figure 1 shows that the linear forms of equations 4 and 5 are supported by our data. Thus, our detailed results for three solid anesthetics are consistent with the critical volume hypothesis, just as previous detailed studies with gases have been shown to be.[3,15] However, the slopes for the gaseous and non-gaseous agents in figure 1 are unexpectedly found to differ by a factor of nearly 2. In tadpoles, on the other hand, our analysis of some preliminary data[12] suggests that halothane and alphadione pressure-reverse identically. Further quantitative work with tadpoles would be useful to resolve this point because of the simplicity of the pharmacokinetics. In the mouse we cannot rule out the possibility that pressure influences the pharmacodynamics of the intravenous agents, perhaps by changing the proportion bound to plasma protein. On the other hand, the membrane/buffer partition coefficient of pentobarbital is almost independent of pressure,[14] so that partial displacement of the barbiturate from a putative membrane site of action would not explain the greater pressure reversal observed. Nor would the non-ideal behavior of nitrogen and argon cause deviations of more than 10 per cent.[3] If, on the contrary, we do assume that the explanation for this slope difference is contained solely within equations (4 and 5), then we may distinguish two limiting cases. First, we may assume the two classes of agent act at the same site; it then follows that E_{50} must be smaller for the non-gaseous than for the gaseous agents. Second, we may assume different sites of action, with the non-gaseous agents' site showing lower solubility, x, and hence E_{50}, and/or greater compressibility, to yield the observed result. The correct interpretation may, of course, lie between these limits. More detailed experiments will clearly be necessary to settle these points, and to determine whether this slope anomaly is compatible with the critical volume hypothesis, or whether it has some artifactual explanation, as suggested by the data from tadpoles.[12]

Finally, our work also has a bearing on the problem of controlling the hyperexcitability observed in divers breathing helium–oxygen mixtures at great depths. Thus, the current use of nitrogen–helium–oxygen mixtures for divers[25] was suggested on the basis that protection against hyperexcitability could be obtained at pressure without incurring nitrogen narcosis because it would be pressure-reversed.[26] Our present results suggest that a wider variety of depressive agents may be employed for this purpose in a similar manner. Such an increase in the modalities available to treat this high-pressure neurologic syndrome could be an advantage in view of the limited effectiveness of gas mixtures at extreme depths.[26,27]

The authors thank Mr. Paul Wankowicz for help in constructing and maintaining the hyperbaric facility.

Note added in proof. A recent publication shows that the pressure reversal of alphadione also occurs more rapidly than that of gaseous anesthetics. Bailey CP, Green CJ, Halsey MJ, and Wardley-Smith B: J Appl Physiol 43: 183–188, 1977.

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Anesthesiology
49:6–8, 1978

Reprinted from: ANESTHESIOLOGY, Vol. 49, No. 1, July 1978

Naloxone Has No Effect on Nitrous Oxide Anesthesia

Raymond A. Smith, D.Phil., Michael Wilson, B.S.,† Keith W. Miller, D.Phil.‡*

It has been reported that naloxone antagonizes general anesthesia in rats when the tail clamp is used as a painful stimulus to assess anesthesia.[1] The authors' hypothesis is that this antagonism is to the analgesic component of anesthesia only, and that anesthesia assessed by a non-painful stimulus would not be antagonized by naloxone. Therefore, the anesthetic potency of nitrous oxide in mice was measured using loss of the righting reflex as a non-painful stimulus. Naloxone, 2 and 16 mg/kg, intraperitoneally, failed to antagonize nitrous oxide anesthesia measured 14–39 min after injection. Thus, 19 min after injection of naloxone, 2 mg/kg, the nitrous oxide ED_{50} was 1.25 ± 0.060 atm (n = 35), compared with 1.19 ± 0.053 atm (n = 35) after injection of saline solution (control). Following naloxone, 16 mg/kg, the nitrous oxide ED_{50} was 1.18 ± 0.059 atm (n = 35), compared with 1.22 ± 0.059 atm (n = 35) for saline solution. At neither dose of naloxone was the ED_{50} different from the control ED_{50}, a finding that supports the authors' hypothesis. (Key words: Analgesia: measurement. Anesthetics, gases: nitrous oxide. Antagonists, narcotic: naloxone.)

BERKOWITZ AND CO-WORKERS[2] have shown that the dose-related analgesia (measured by the writhing response to intraperitoneal injection of phenylquinone in mice) produced by nitrous oxide is reversed by naloxone. They suggested that the analgesia associated with general anesthesia may be related to the release of endogenous opiates. Finck *et al.* recently studied the effect of naloxone on general anesthesia, based on the possibility that anesthetics may act partly by releasing a morphine-like substance.[1] Using constant doses of halothane, enflurane, or cyclopropane such that about 40 per cent of the animals responded to a tail clamp applied for 30 sec, they found that the average number of animals responding increased from 40 to 70 per cent when naloxone was given. Since analgesia is part of the state of anesthesia, they concluded that naloxone partially antagonizes general anesthesia. We believe this conclusion may be related to the painful stimulus they used to assess anesthesia.

Any measure of anesthesia must involve both a stimulus and a purposeful response. The concentration of anesthetic necessary to block a response increases with the intensity of the stimulus until a plateau of supramaximal stimulation is reached, after which no further increase in dose is needed.[3] In the experiments of Finck *et al.*[1] the physical stimulus (tail clamp) was the same for both naloxone-treated and control animals. However, at a given anesthetic concentration, the perception of this painful stimulus in the central nervous system may have been greater in the naloxone-treated than in the control animals, so that they responded more. We have performed experiments using the rotation of an animal's cage as a nonpainful stimulus. This stimulation of the righting reflexes provides an observable anesthetic end-point that yields ED_{50} values close to those obtained using painful stimuli.[4]

Methods

We used a 34-l steel pressure chamber equipped with a circulating fan, carbon dioxide scrubber, temperature control device, and motors to rotate the cages.[5] Eight mice received intraperitoneal injections of naloxone hydrochloride, 2 or 16 mg/kg, and eight control mice received injections of physiologic saline solution. The latter dose of naloxone is at least 32 times the dose that was found to antagonize completely the analgesic effect of either fentanyl alone or fentanyl combined with droperidol for at least 30 min when injected intraperitoneally in mice.[6] Seven each of the naloxone-treated and the control mice were placed in individually marked wire mesh cages. Two sets of seven cages were mounted in front of different windows in the pressure chamber. Chamber temperature was monitored and adjusted to maintain the rectal temperatures of the two remaining restrained mice between 36.5 and 38 C. The chamber was sealed and the oxygen partial pressure increased to 0.5 atm. Nitrous oxide was then immediately added to the desired partial pressure, a total of 9 min after injection. The rolling response was measured 5, 10, 20, and 30 min after exposure to nitrous oxide. Two observers, who were unaware of the treatment each mouse had received, each recorded the response of seven animals. The cages were rotated at 4 rpm. All mice that rolled over completely during more than one revolution of a five-revolution sequence were scored 0. Mice remaining upright or falling over during one revolution only were scored 1. We used new groups of mice for each of five doses of nitrous oxide (0.98, 1.13,

* Research Fellow, Harvard Medical School.

† Research Assistant, Massachusetts General Hospital.

‡ Associate Professor of Pharmacology in the Department of Anesthesia, Harvard Medical School.

Received from the laboratories of the Department of Anesthesia, Harvard Medical School at the Massachusetts General Hospital, Boston, Massachusetts 02114. Accepted for publication January 6, 1978. Supported in part by an Office of Naval Research contract (N00014-75-C-0727) with funds provided by the Naval Medical Research and Development Command and in part by a grant from the National Institute of General Medical Sciences (GM-15904). Dr. Miller is supported by a Public Health Service Career Development Award (GM-00199) from the National Institute of General Medical Sciences.

Address reprint requests to Dr. Miller.

0003-3022/78/0700/0006 $00.60

TABLE 1. ED_{50} Values for Loss of Righting Responses during Exposures to Five Doses of Nitrous Oxide in Control and Naloxone-treated Mice

	Time after Injection of Naloxone (Min)	Time after Nitrous Oxide Exposure (Min)	Saline Solution $ED_{50} \pm SE$ (Atm) n = 35 (Seven Animals at Each N_2O Dose)	Naloxone $ED_{50} \pm SE$ (Atm) n = 35 (Seven Animals at Each N_2O Dose)	Significance of Difference Between ED_{50} Values
Naloxone, 2 mg/kg, ip	14	5	1.08 ± 0.036	1.11 ± 0.034	$P = 0.92$
	19	10	1.19 ± 0.053	1.25 ± 0.060	$P = 0.90$
	29	20	1.20 ± 0.057	1.31 ± 0.077	$P = 0.84$
	39	30	1.16 ± 0.035	1.23 ± 0.039	$P = 0.82$
Naloxone, 16 mg/kg, ip	14	5	1.17 ± 0.095	1.05 ± 0.107	$P = 0.38$
	19	10	1.22 ± 0.059	1.18 ± 0.059	$P = 0.60$
	29	20	1.25 ± 0.059	1.29 ± 0.061	$P = 0.60$
	39	30	1.18 ± 0.060	1.27 ± 0.062	$P = 0.14$

1.17, 1.22, and 1.35 atm for the 2 mg/kg naloxone series of experiments and 1.01, 1.14, 1.26, 1.40, and 1.52 atm for the 16 mg/kg naloxone series).

The resulting five-point dose–response curves were analyzed by logit methods.[7] First, the curves obtained with naloxone and with saline solution were analyzed allowing their slopes to vary independently. Since there was no significant difference between the two slopes, a second analysis assuming a common slope for the two curves was performed to obtain ED_{50} values with their standard errors.

Results

The ED_{50} values for the control and naloxone-treated mice were not significantly different (table 1). A t test for paired data of the individual responses in each group at each dose also failed to show significant differences between the groups. The ED_{50} values 5 min after exposure showed evidence of a rapidly developing acute tolerance, previously reported by Smith *et al.*§ The later ED_{50} values were higher than the 5-min ED_{50} values in both control and naloxone-treated mice. Therefore, we conclude that naloxone had no effect on the development of acute tolerance.

Discussion

We must consider the possibility that the ineffectiveness of naloxone in our experiments resulted from its rapid removal from the brain. Ngai *et al.*[8] have shown that in rats given naloxone, 5 mg/kg, intravenously drug levels decayed with a half-life of 30 min in serum, and that brain levels paralleled serum levels. Furthermore, in mice, Smith[6] has shown that as little as 0.5 mg/kg naloxone administered intraperitoneally completely reversed both the analgesic and the respiratory depressant effects of fentanyl (0.4 and 0.8 mg/kg), but that there was an observable return of analgesia at 45–60 min. It thus seems improbable that ineffective levels of naloxone were present in our experiments, particularly at the highest dose used.

Thus, our results show that naloxone has no effect on the ability of nitrous oxide to prevent mice from perceiving spatial disorientation, whereas Berkowitz *et al.*[2] have shown that naloxone reverses nitrous oxide-induced analgesia assessed by a noxious writhing test. There is no contradiction in these observations, since the latter deals purely with the analgesic effect of nitrous oxide whereas the former does not involve any anti-nociceptive response. Classically, general anesthesia consists of several components, which include loss of consciousness, amnesia, analgesia, and depression of reflexes. The experiments of Finck *et al.*[1] measured the ability of rats that had central nervous system depression to respond to a painful stimulus. This provides a composite end-point that includes analgesia and, probably, loss of consciousness and the depression of reflexes. The rolling response, which we used, is also a composite end-point, which probably includes both loss of consciousness and depression of reflexes. Although we do not know what proportions of these components of anesthesia are represented by the tail clamp and the rolling response end-points, the three sets of observations are consistently explained if naloxone can antagonize the analgesic but not the other components of anesthesia caused by nitrous oxide.

Several studies have suggested that naloxone may also antagonize certain effects of other non-opiate central nervous system depressants. Blum *et al.* have reported that ethanol-induced sleeping time in mice is decreased significantly by naloxone, 5 mg/kg, intraperitoneally.[9] Fürst *et al.* showed that naloxone, 1 mg/kg, delayed the onset and decreased the duration of pentobarbital- or methohexital-induced loss of

§ Smith RA, Winter PM, Smith M, et al: Rapidly developing tolerance to an acute exposure of nitrous oxide (abstr. American Society of Anesthesiologists Annual Meeting, 1976, pp 313–314.

righting reflex (sleeping time) in rats.[10] However, without knowledge of the brain levels of the depressant drugs used in these experiments, and because of the problems of associating changes in sleeping time with shifts in dose–response curves, it is difficult to assess the true difference between results in naloxone-treated and control animals in these studies. In our studies the inspired doses of nitrous oxide and the rectal temperatures of the mice were kept constant, and thus brain levels of nitrous oxide would be constant and equal in both naloxone-treated and control mice. Thus, even though anesthetics and other non-opiate central nervous system depressant drugs might release an opiate-like factor in the brain,[1] there is at present no unequivocal evidence that this contributes to any other than the analgesic component of the state of general anesthesia.

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BBA 78067

CHOLESTEROL MODULATES THE EFFECTS OF MEMBRANE PERTURBERS IN PHOSPHOLIPID VESICLES AND BIOMEMBRANES

KAM-YEE Y. PANG and KEITH W. MILLER

Departments of Pharmacology and Anesthesia, Harvard Medical School and Massachusetts General Hospital, Boston, Mass. 02114 (U.S.A.)

(Received December 2nd, 1977)

Summary

The order parameter of spin-labeled phosphatidylcholine vesicles has been shown to increase upon incorporation of cholesterol, cannabinol, chlorpromazine and pentobarbital. Cannabinol was as effective on a mole basis as cholesterol in increasing the order parameter of 5-doxyl stearic acid in phosphatidylcholine: 4% phosphatidic acid bilayers at low concentrations. The average increase in order parameter with chlorpromazine and pentobarbital was two to three times less than that of cholesterol. Relative to cholesterol these compounds were less effective at ordering 1-acyl-2[8(4,4-dimethyloxazolidine-*N*-oxyl)] palmitoyl phosphatidylcholine.

The ordering effect with any given membrane perturber became smaller when increasing amounts of cholesterol were incorporated in the phospholipid bilayers until a disordering effect was finally observed. The cholesterol composition at which this cross-over from ordering to disordering occurred varied with the perturber, being 26 mole % for chlorpromazine, 23 mole % for cannabinol and 14 mole % for pentobarbital. The ability of cholesterol itself to increase the order parameter of the bilayer was decreased in the presence of these perturbers.

These compounds may exert their ordering effect on the interfacial region of phospholipid bilayers in an analogous manner to cholesterol. However, at higher cholesterol contents their additional ordering effect is more than counterbalanced by a weakening of the cholesterol-acyl interaction and a net disordering effect results.

In biological membranes a similar role for cholesterol in modulating the effect of perturbers was observed. Cannabinol decreased the order of erythrocyte membranes but increased that of mitochondrial membranes, while octanol disordered both of these biological membranes.

Abbreviations: PC (7,6), 1-acyl-2[8(4,4-dimethyloxazolidine-*N*-oxyl)] palmitoyl phosphatidylcholine; PC (10,3), 1-acyl-2[5(4,4-dimethyloxazolidine-*N*-oxyl)] palmitoyl phosphatidylcholine.

Introduction

Cholesterol is a major component in mammalian membranes and yet its content varies from one membrane to another; it ranges, for example, from 40 mole % in myelin to 6 mole % in mitochondria [1]. The exact function of cholesterol in the membrane is still unknown, but biophysical studies on model membranes show that one role of cholesterol in membranes is to regulate the degree of order and mobility of the acyl chains of lipids, i.e., above the transition temperature of the phospholipid, cholesterol reduces the mobility of the acyl chain while below the transition temperature, it has the opposite effect. Cholesterol thus acts as a modulator of the packing of the acyl chains of phospholipids.

On the other hand many studies [2—4] have shown that small lipophilic molecules fluidize membranes. Many of these compounds are pharmacological agents such as general and local anesthetics and their ability to fluidize membranes has been related to their pharmacological potency. However, more recently a few studies have shown that lipophilic molecules may also exert a condensing effect on lipid bilayers. Thus, cannabinol increases the order parameter of dipalmitoyl phosphatidylcholine-cholesterol bilayers [5], and local anesthetics have a similar effect on decholesterolized ox-brain lipids [6]. In a preliminary communication [7] we have shown that pentobarbital and some other anesthetics may exert either a condensing or a disordering effect on phosphatidylcholine bilayers depending on their cholesterol content. Thus, under some circumstances these compounds mimic the effect of cholesterol in reducing the mobility of the acyl chains in phospholipid bilayers. In this study we compare the ability of a number of compounds to order phospholipid bilayers and we examine in detail the role of the cholesterol content of bilayers in modulating their ability to be either ordered or disordered. We also show that our conclusions are applicable to biological membranes.

Materials and Methods

The effect of the lipid-soluble compounds on membrane structure was generally monitored from the electron spin resonance (ESR) spectra of 5-doxyl stearic acid (Synva, Calif.) and 1-acyl-2[8(4,4-dimethyloxazolidine-*N*-oxyl)] palmitoyl phosphatidylcholine (PC (7,6)) label which was synthesized in this laboratory by Dr. M. Pringle according to the method of Hubbell and McConnell [8]. Egg yolk phosphatidylcholine and phosphatidic acid were from Lipid Products, Surrey, U.K. and used without further purification. Cholesterol (Sigma) was recrystallized in methanol. Chlorpromazine hydrochloride and pentobarbital were from Sigma. Octanol was purchased from Applied Science, State College, Pa. One sample of cannabinol was a gift from the National Institute on Drug Abuse, and was 98% pure. Another was purchased from Poly Science, Warrington, Pa at 95% purity and further purified by thin layer chromatography [9].

Stock solutions (36 mg/ml) of phosphatidylcholine: 4% phosphatidic acid containing various mole percentages of cholesterol were made up in chloroform/methanol (9 : 1, v/v) to avoid variation between experiments. 0.5 ml of

stock solution was mixed with the spin label in methanol and with drugs in chloroform/methanol or ethanol and dried down in a rotatory evaporator. Residual solvent was removed by pumping on a vacuum line for at least 2 h. Tris buffer (0.01 M, pH 7) in 0.15 M KCl was added and liposomes were formed by vigorous vortexing for 1 min. The final concentration of lipid was 15—25 mg/ml, with spin label constituting about 1 mole % of the lipid. Drugs were equilibrated with liposomes for up to 24 h at 25°C before being sealed in 1 mm glass capillaries. All the drugs in the study are highly lipophilic, for example, the partition coefficient of octanol is 152 in erythrocytes [10] and 670 in dipalmitoyl phosphatidylcholine [11] and, thus, are expected to be mainly concentrated in the lipid phase. Spectra were recorded on either a Varian E-9 or E-109 electron spin resonance spectrometer operating at 9.5 GHz with the variable temperature control unit set at 23°C or 25°C. Order parameters, S, were calculated from the spectra according to the method of Hubbell and McConnell and the $T_\perp$ value was corrected by the method of Gaffney [8,12].

Mitochondria from rat liver were prepared using the method of Parsons and Williams [13]. Erythrocyte ghosts were prepared from out-dated human blood according to the procedures of Dodge et al. [14]. The protein concentration of membranes was determined by the method of Lowry [15]. To label the membranes, 1 ml of a membrane solution was added to a test tube containing an appropriate amount of 5-doxyl stearic acid label previously deposited in a thin film, and was incubated overnight at 4°C. The spin-labeled membrane solutions were spun down (9750 × g for 10 min for mitochondria; 20 000 × g for 30 min for erythrocyte ghosts) and resuspended in Tris buffer. Aliquots of labeled mitochondrial membranes were incubated for 1 h at 23°C with the desired amount of cannabinol or octanol, which were previously deposited on test tubes. For erythrocyte ghosts, the incubation with drugs was usually carried out at 4°C overnight. ESR spectra were obtained at 23°C.

Results

The order parameter, S, in phosphatidylcholine: 4% phosphatidic acid bilayers increases when either cholesterol, cannabinol, chlorpromazine or pentobarbital are added (Fig. 1, A and B). Similar results have been reported for cholesterol in phosphatidylcholine bilayers [16—21] and for pentobarbital and chlorpromazine in decholesterolized ox-brain lipids [6]. None of the other compounds produced such a large absolute ordering as cholesterol. Cannabinol was the strongest orderer of the other three. It ordered 5-doxyl stearic acid as strongly as cholesterol on a mole basis, but the effect appeared to saturate above 17 mole %. No such saturation was observed up to 34 mole % by PC (7,6), possibly reflecting different interactions at the interface and the interior of the bilayer. There is also a suggestion, particularly with 5-doxyl stearic acid, of curvature in some of the other data, but the effect is not large compared to our errors and because of the difficulties of solubilizing these compounds we did not pursue it in more detail. Nor did we find any consistent and reproducible non-linearities in other experiments even at high concentrations (e.g. Figs. 2 and 5). With these reservations we have assumed linearity in our analysis.

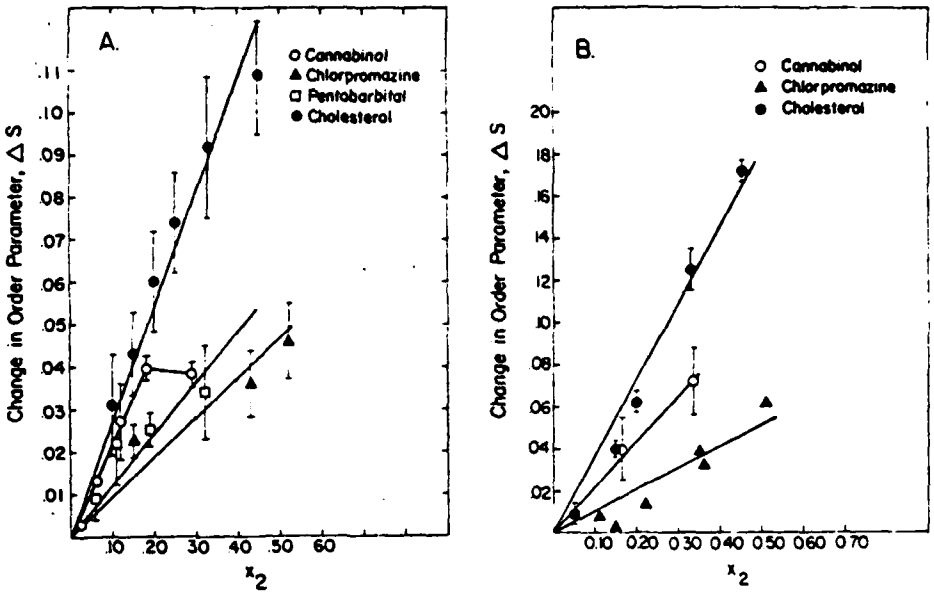

Fig. 1. A. Change in order parameter (ΔS) of spin labeled 5-doxyl stearic acid in phosphatidylcholine: 4% phosphatidic acid vesicles containing various mole fractions of cholesterol, cannabinol, chlorpromazine and pentobarbital. The slopes ($\Delta S/X_2$) were as follows: cholesterol, 0.27 ± 0.01 (S.D.), cannabinol, 0.23 ± 0.03; chlorpromazine, 0.09 ± 0.01; pentobarbital, 0.12 ± 0.01. Error bars are standard deviations, other points represent single experiments, and the regressions were weighted by the number of points. This procedure is followed in all subsequent figures. B. The corresponding results with PC (7,6) were: cholesterol, 0.37 ± 0.02; cannabinol, 0.22 ± 0.01; chlorpromazine, 0.10 ± 0.01. The mean order parameter for control vesicles with 5-doxyl stearic acid was 0.599 ± 0.009; with PC (7,6) was 0.499 ± 0.005.

Cholesterol increases the order of 5-doxyl stearic acid less than that of PC (7,6) (Fig. 1, A and B), as has been found by previous workers [17,22]. However, recent deuterium magnetic resonance studies show that cholesterol causes a uniform ordering of the acyl chains in this region [21,23,24]. A similar discrepancy is found between the two methods when the fluidity gradient is considered [21]. An additional uncertainty in our experiments is the location of 5-doxyl stearic acid compared to PC (7,6). We attempted to use 1-acyl-2[5(4,4-dimethyloxazolidine-*N*-oxyl)] palmitoyl phosphatidylcholine (PC (10,3)) instead but it gave such a high order parameter in the phospholipid bilayers that additional ordering was difficult to detect. Thus, comparisons between the absolute effects of perturbers on our two labels should be made with caution. On the other hand, these considerations are unlikely to invalidate our results when relative ordering effects are considered on a given label.

Relative to cholesterol none of these compounds ordered PC (7,6) as much as they did 5-doxyl stearic acid. Therefore, their relative ordering effects are probably strongest near the bilayer surface. Unfortunately 12-doxyl stearic acid yields too fluid a spectrum for order parameters to be measured. However, in one experiment 24 mole % of pentobarbital gave a spectrum identical to the control, tending to confirm the impression that the ordering effect gets weaker

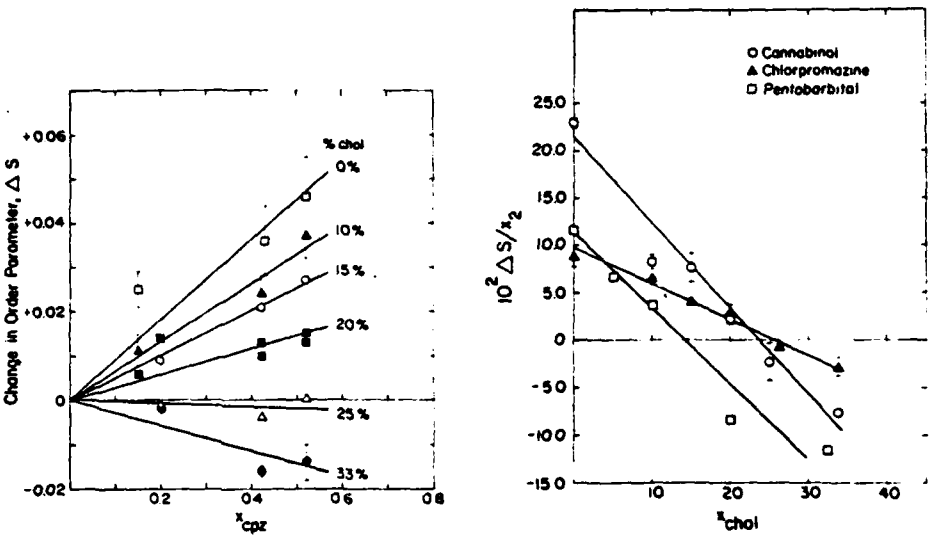

Fig. 2. The effect of chlorpromazine on the change in order parameter (ΔS) in phosphatidylcholine: 4% phosphatidic acid containing various mole % of cholesterol. X_{cpz} is chlorpromazine/chlorpromazine + phospholipid. Percent cholesterol is 100 (cholesterol/cholesterol + phospholipid).

Fig. 3. The effect of cholesterol on the change in order parameter per mole of membrane perturbers in phosphatidylcholine: 4% phosphatidic acid vesicles. The mole percentage of cholesterol at the cross-over point, ($\Delta S = 0$) were as follows: cannabinol, 23 ± 1.0; chlorpromazine, 26 ± 1.2; pentobarbital, 14 ± 1.8. X_{chol} is cholesterol/cholesterol + phospholipid.

further from the interface. The remainder of our work was carried out with 5-doxyl stearic acid because it gives the largest effects.

When one of the membrane perturbers was added to bilayers containing cholesterol the magnitude and sign of the change in order parameter was dependent on the cholesterol content. Thus, Fig. 2 shows that chlorpromazine orders bilayers with cholesterol content up to 20 mole %, but disorders those with greater than 25 mole % cholesterol. Similar results (not shown) were obtained with cannabinol and pentobarbital. If the change in order parameter per unit concentration of perturber in the bilayer (i.e., the slope in Fig. 2) is plotted versus the mole % of cholesterol in the lipid, the cholesterol content at which ΔS is zero may be defined. In Fig. 3 this cross-over composition of cholesterol is seen to be 26 mole % for chlorpromazine, 23 mole % for cannabinol and 14 mole % for pentobarbital.

In biological membranes a similar role for cholesterol in modulating the effects of perturbers was observed (Fig. 4). Cannabinol decreased the order of erythrocyte ghost membranes which have about 40 mole % cholesterol [1], but increased that of mitochondrial membranes which have an average lipid composition of 6 mole % cholesterol [1]. Previously we have shown [7] that octanol disorders phosphatidylcholine: 4% phosphatidic acid bilayers regardless of cholesterol content. Fig. 4, A and B, show that it also disorders both the biological membranes examined.

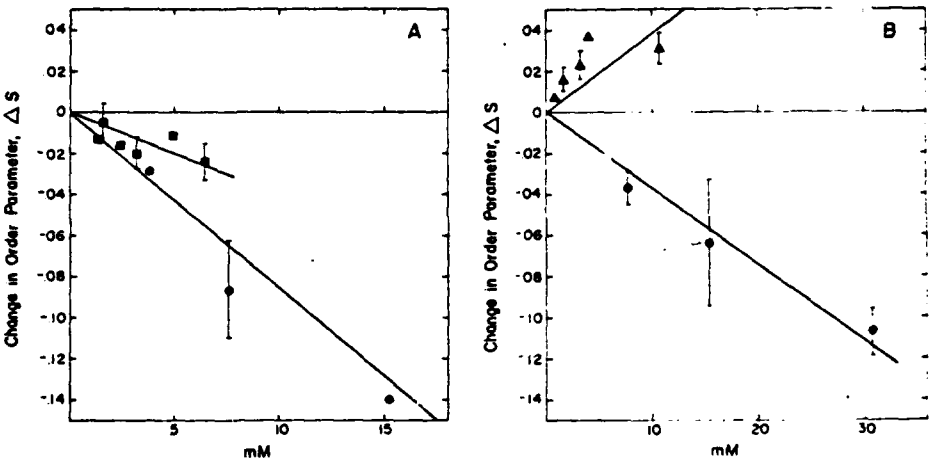

Fig. 4. Effect of increasing total concentrations of cannabinol (■) and octanol (●) on the change in order parameter (ΔS) of 5-doxyl stearic acid in A: *erythrocyte membranes, protein concentrations* 1—4 mg/ml. The mean order parameter of the control was 0.714 ± 0.033. B: mitochondrial membranes, protein concentrations 23 mg/ml. The mean order parameter of the control was 0.598 ± 0.012.

Discussion

The change in order produced by cholesterol probably reflects the orientation of the rigid steroid nucleus parallel to the phospholipid acyl chains, with the 3 β-hydroxyl group located in the interfacial region [21,24—26]. All the compounds which order phospholipid bilayers [4—7] also contain chemical ring structures, so they may act in a manner analogous to cholesterol. However, one might expect that they would produce a less extensive ordering than cholesterol because of their less favorable geometry. Thus, both chlorpromazine and cannabinol have three fused rings, but those of the former are folded about the N-S axis at 139.4° [27], and the presence of a charge may also limit its penetration into the bilayer. On the other hand, the rings of cannabinol should not deviate seriously from coplanarity, but the proximity of the hydroxyl and pentyl substituent groups should mitigate against orientation of the long axis of the fused ring structure parallel to the acyl chains. These considerations are consistent with our finding that these perturbers are less effective orderers at the eighth than at the fifth carbon. Similarly the deuterium magnetic resonance data show that the ordering effect of cholesterol declines beyong the 12 carbon [21], and it is possible with the other perturbers examined here that their ability to order might fall off even more rapidly. Preliminary studies of fluorescence depolarization with 1,6-diphenyl hexatriene in this laboratory show a decrease in microviscosity in pentobarbital-phosphatidylcholine liposomes. This result lends support to the suggestion that it is possible that our perturbers simultaneously order the ester terminal of the acyl chains whilst disordering the methyl terminal. Deuterium magnetic resonance studies could confirm this prediction.

Interactions in the system containing phospholipid-cholesterol-perturber are

more complex. Thus, the ordering effects of cholesterol and perturber are clearly not additive (Figs. 2 and 3). The favorable stereochemistry, which allows a strong van der Waals interaction between cholesterol and the acyl chains [21,24–26], will tend to be disrupted by the presence of the perturber in the same region of the bilayer. The net effect of the perturber on the packing of phospholipid-cholesterol bilayers will reflect the balance between its own ordering effect on the acyl chain and its disruptive effect on the acyl-cholesterol interaction. As the cholesterol content of the bilayer is increased, cholesterol-perturber interactions become more frequent. Eventually the resultant disruption of acyl-cholesterol interactions outweigh the ordering effect of the perturber and a cross-over to a net disordering effect is observed (Fig. 2). This apparently may occur at any cholesterol composition, depending only on the balance of these effects and not on any discrete phospholipid to cholesterol ratio. Pentobarbital, the weakest orderer, crosses over at the lowest cholesterol content (Fig. 3). In phospholipid bilayers, where only the perturber's ordering effect is important, larger changes in order parameter occur than in high cholesterol membranes where the changes result from a balance between two opposing effects (Fig. 2). Chlorpromazine and cannabinol cross-over from ordering to disordering at similar cholesterol contents even though chlorpromazine, like pentobarbital, is a less effective orderer of phospholipid bilayers. This results from the less steep slope exhibited by chlorpromazine in Fig. 3 and may reflect the additional role of chlorpromazine's positive charge which would limit its ability to penetrate fully into the region where cholesterol exerts its ordering effect.

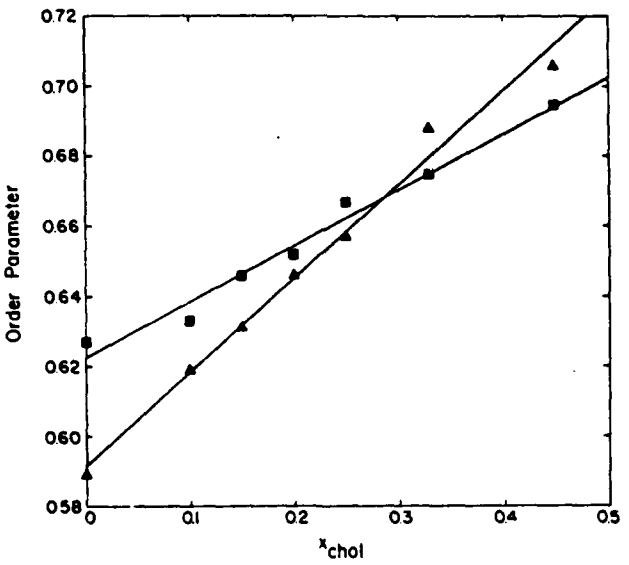

Fig. 5. Effect of cholesterol (chol) on the order parameter of phosphatidylcholine: 4% phosphatidic acid liposomes in the absence (▲) and in the presence, (■) of 17 mole % cannabinol. The slopes of the lines are 0.270 ± 0.001 and 0.160 ± 0.001, respectively.

This ability of perturbers to reduce the effectiveness of cholesterol to order bilayers is clearly illustrated in Fig. 5. Here cholesterol orders a phospholipid bilayer containing 17 mole % of cannabinol 1.7 times less effectively than in the unadulterated bilayer.

Thus, we may conclude that, in addition to its well known property of regulating membrane fluidity, cholesterol also modulates the effect of membrane perturbers in both lipid bilayers and biological membranes. If more extensive studies confirm this there are a number of interesting corollaries. Thus, many authors [28—31] have emphasized the possible relationship between the action of anesthetics and their ability to fluidize membranes. Our work here on biomembranes, together with previous work on general anesthetics in lipid bilayers [7], suggests that for this hypothesis to be correct the model must be restricted to biomembranes containing a high proportion of cholesterol. This would be consistent with the known composition of most neural membranes [32].

Changes in fluidity related to cholesterol content have been noted upon malignant transformation of normal lymphocytes [33], the fusion of muscle cells [34] and the complement lysis of antibody-sensitized cells with normal rabbit serum [35]. If there is a causal relationship between these phenomena and membrane fluidity, then the ability to selectively modify biomembrane fluidity by pharmacological agents may not be without application.

Acknowledgements

Keith W. Miller is supported by a U.S. Public Health Service Research Career Development Award from the National Institute of General Medical Sciences. This work was also supported by a contract from the Office of Naval Research, Physiological Branch, with funds provided by the Naval Research and Development Command. The authors wish to thank Dr. G. Gergeley and Dr. D. Thomas, Boston Biomedical Research Institute, for the use of their electron spin resonance equipment.

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MOLECULAR PHARMACOLOGY, 14, 950–959

Pressure Resolves Two Sites of Action of Inert Gases

KEITH W. MILLER,[1] MICHAEL W. WILSON, AND RAYMOND A. SMITH

Departments of Pharmacology and Anaesthesia, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114

(Received January 27, 1978)
(Accepted April 14, 1978)

SUMMARY

MILLER, KEITH W., WILSON, MICHAEL W. & SMITH, RAYMOND A. (1978) Pressure resolves two sites of action for inert gases. *Mol. Pharmacol.*, **14**, 950–959.

The effect of pressure upon the potency of two pharmacological effects of inert gases has been studied in mice. In one series of experiments the effect of high pressures of helium on the anesthetic potency of nitrogen, argon, nitrous oxide, carbon tetrafluoride and sulfur hexafluoride was studied up to pressures of 183 atm. Pressure increased the ED_{50} for loss of righting reflexes by 36% at 100 atm on average. In the other experiments we measured the ability of these inert gases to raise the ED_{50} pressure at which pressure-induced hyperexcitability (spasms) was observed. Subanesthetic partial pressures of all the gases raised the ED_{50} pressure for spasms significantly. These data were used to test the two hypotheses that anesthesia results when anesthetics expand some hydrophobic phase by a critical amount, while the hyperexcitability occurs when pressure reduces the volume of some hydrophobic phase by a critical amount (the critical volume hypothesis). Theoretical calculations show that both sets of data are consistent with their respective hypotheses. The site at which the inert gases exert their anti-hyperexcitability effect is much more compressible and has a slightly lower solubility parameter than the site for anesthesia.

INTRODUCTION

The traditional lipid solubility theories of general anesthetic action (1, 2) have had to be modified in recent years to account for the remarkable antagonism of general anesthesia by pressure per se (3, 4). The currently accepted form of the lipid theory is the critical volume hypothesis, which states that anesthesia occurs when the volume of a hydrophobic region is caused to expand beyond a certain critical volume by the absorption of molecules of an inert substance. Pressure opposes this volume change and so reverses the anesthesia (5). This hypothesis has been shown to be quantitatively consistent with pressure reversal data for a number of anesthetics in newts (5). Pressure reversal also has been demonstrated for a variety of agents in tadpoles (6) and for conduction block in the squid giant axon (7, 8). The question then arises whether the critical volume hypothesis describes a general mechanism of anesthetic action which may occur at different sites of action within neural tissue, and if so whether the physical parameters required to describe such sites will vary from site to site. In this paper we have examined the interaction between unreactive gases

This research was supported in part by a National Institute of General Medical Sciences Research Center Grant (GM-15904-10) and in part by an Office of Naval Research Contract (N00014-75-C-0727) with funds provided by the Naval Medical Research and Development Command.

[1] Keith W. Miller is a Research Career Development Awardee of N.I.G.M.S. (GM 00199).

0026-895X/78/0145-0950$02.00/0

and pressure in mice when the gases act either as general anesthetics or as agents which ameliorate the high pressure neurological syndrome (9). By a suitable choice of gases, specifically the inclusion of fully fluorinated compounds (10), we show that these two pharmacological properties of the gases are mediated by a hydrophobic site or sites with rather similar solvent properties. However, the isothermal compressibility parameter, which describes the variation of pharmacological potency with pressure, differs by half an order of magnitude for these two sites. Thus, we have differentiated two pharmacological sites of action of inert gases in a single species of mammal on the basis of the effects of pressure. A preliminary analysis of available data had previously shown this to be the case when different species of mice were involved (11), although this conclusion was questionable because insufficient data were then available to define the solvent properties of the site of action. In a number of other studies sites of action of volatile agents have been distinguished on the basis of solvent properties (2, 12).

The application of the critical volume hypothesis to the high pressure neurological syndrome has been proposed previously (11). It states that the high pressure neurological syndrome occurs when some hydrophobic region has been compressed beyond a critical amount by the application of pressure. Absorption of an inert gas will cause an expansion which compensates for such compression and raises the threshold pressure for symptoms. The high pressure neurological syndrome is a complex of symptoms that are observed when mammals are compressed either hydraulically (13, 14) or in helium-oxygen atmospheres (15). At moderate pressure it manifests itself as tremor of the limbs but as the pressure is further raised rhythmic spasms, clonic, and then tonic convulsions occur. In this paper we consider the effect of inert gases on the threshold pressure for spasms, which is one of the earliest objectively definable end points. (The tremors which occur at lower pressure were too difficult to distinguish from the fasciculation caused by moderate doses of anesthetics.)

METHODS

Male CD-1 mice (Charles River) weighing 20–30 g were used in all experiments. Measurements of anesthetic potency were performed in a 34 liter steel hyperbaric chamber equipped with two large windows, a temperature control system and moisture and carbon dioxide scrubbers. Complete details have been published previously (16).

The level of anesthesia was determined by testing the ability of each mouse to remain upright when its cylindrical wire mesh cage was rotated at 4 rpm for five complete revolutions; a score between 0 and 5 was assigned (16). A group of seven mice was exposed at each window. Two additional mice with rectal thermistors were placed between these groups, and the chamber temperature adjusted to maintain rectal temperature at 37 ± 1°C. The partial pressure of oxygen was maintained in the range of 0.3 to 1.0 atm.

Mice were examined for the high pressure neurological syndrome in separate experiments. Two groups of five mice were used in each experiment, together with two additional rectal temperature controls. After addition of the anesthetic gas, helium was admitted to the chamber at a constant compression rate of 60 atm per hour and the animals continuously monitored for the symptoms of the high pressure neurological syndrome. Complete spasms were defined as rhythmic tensing and relaxing of all muscle groups but not of sufficient severity to cause the animal to lose its upright posture (17). These spasms generally were observed to appear after the onset of coarse whole body tremors but before generalized clonic convulsions involving loss of upright posture (15, 17).

During the anesthesia experiments the number of mice responding was determined as a function of anesthetic partial pressure at a series of given total pressures. At each total pressure a dose response curve was obtained and analyzed on a digital calculator using the method of Waud for quantal responses (18). Since each animal was used at several doses on each curve, the number of animals at each dose was weighted so that the sum for all doses equalled the

number of animals actually employed. Pressure-response curves for the high pressure neurological syndrome were obtained for each gas mixture and analyzed as above, but since each animal responded once only weighting was unnecessary. In about half of these experiments complete spasms were not observed in all animals, but all the animals were included in the cumulative pressure-response curve in order to avoid biasing the analysis with the most susceptible animals.

Helium (99.995% pure) and nitrous oxide (98%) were obtained from Ohio Medical Products, Wisconsin; nitrogen (99.9%) and oxygen (99.6%) were from Medical Technical Gases, Massachusetts; argon (99.995%) and sulfur hexafluoride (99.8%) were from Matheson Gas Products, New Jersey; and hexafluoroethane (99.6%) and carbon tetrafluoride (99.7%) were from Dupont-de-Nemours EI and Co., Delaware.

RESULTS

Experimental

ED_{50} values for the loss of righting reflex in mice are listed in Table 1 with their standard errors and scale parameters which provide a measure of the slope of the dose response curves (18). Some of the data for nitrogen and argon have been published previously by us but are reproduced here for completeness (16). The ED_{50}'s in the absence of helium agree well with earlier data (19, 20). The value for carbon tetrafluoride is higher than that obtained under less well controlled conditions (19), while that for nitrous oxide is lower than most (19–21), but not all (22), literature values. Sulfur hexafluoride exhibited a low therapeutic index and it was impossible to obtain meaningful results unless some helium was added. However, the value obtained agrees closely with previous work (20). Similarly, hexafluoroethane caused marked respiratory distress. By increasing the partial pressure of oxygen to two atmospheres and lowering the rectal temperature by 0.5°C, we determined an ED_{50} close to that reported by Miller et al. (20).

There were no consistent and significant trends in the slopes of the dose-response curves with increasing pressure (Table 1). This contrasts with intravenous anesthetics, where a small but significant increase in slope with pressure has been reported (16). Comparison of the ED_{50} at one atmosphere (extrapolated where necessary) to that at 100 atm reveals the following percentage increases: N_2 33; CF_4 34; Ar 33; SF_6 36; N_2O 42. These values agree closely with those obtained independently for N_2O, N_2 and Ar (21, 23). For each agent plots of ED_{50} as a function of pressure were linear, and introduction of a quadratic term in the regression gave no significant improvement in fit (F-test).

Pressure per se causes marked effects above about 140 atm (13–15); consequently most of our measurements were obtained at lower pressures. With the densest gases

TABLE 1

Variation of ED_{50} values for the loss of righting reflex in mice as a function of pressure

Anesthetic	Total pressure	ED_{50} ± SE	Scale parameter ± SE	N
	atm	*atm*		
N_2O	2.2	1.22 ± 0.048	−9 ± 4.0	56
	30	1.32 ± 0.033	−17 ± 6.6	56
	40	1.35 ± 0.022	−22 ± 6.3	70
	50	1.30 ± 0.074	−9 ± 4.3	70
	80	1.61 ± 0.059	−12 ± 5.3	41
	90	1.61 ± 0.038	−17 ± 5.8	41
N_2	40[a]	38.9 ± 0.94	−12 ± 3.8	63
	81[a]	45.8 ± 0.68	−30 ± 14	26
	101	47 ± 1.1	−20 ± 11	26
	121[a]	48.9 ± 0.52	−50 ± 20	26
	141	50.4 ± 0.93	−26 ± 9.7	26
Ar	19[a]	18.1 ± 0.67	−11 ± 4.6	35
	81[a]	21.4 ± 0.78	−14 ± 8.7	17
	124[a]	24.2 ± 0.59	−32 ± 16	14
	128	26 ± 1.6	−11 ± 7	14
	131	23.7 ± 0.77	−20 ± 11	17
	183	27.1 ± 0.67	−25 ± 15	14
CF_4	27	26 ± 1.0	−12 ± 5.2	26
	60	30 ± 1.4	−10 ± 6.7	26
	99	33 ± 1.2	−20 ± 11	14
	140	35 ± 2.9	−11 ± 13.3	12
SF_6	12	5.58 ± 0.086	−16 ± 3.1	119
	20	6.08 ± 0.072	−29 ± 6.5	80
	40	6.42 ± 0.11	−19 ± 6.8	47
	50	6.6 ± 0.08	−28 ± 10.4	48
	60	6.5 ± 0.16	−15 ± 19.3	33
	70	6.8 ± 0.11	−21 ± 8.5	43
C_2F_6	23	17.1 ± 0.39	−11 ± 3.2	84

[a] Data previously reported (16).

studied (SF_6 and C_2F_6), the stress of the hyperbaric environment further limited the accessible pressure range (Table 1). In the case of argon, however, by using slow compression rates we were able to demonstrate pressure reversal at pressures up to 183 atm. Thus, although the hyperbaric environment undoubtedly introduces significant problems in mammalian studies, necessitating close control of environmental conditions, satisfactory quantitative data are obtainable and reproducibility between laboratories is fair (21, 23). The complete spasms threshold pressures are given in Table 2. The threshold pressure in helium was found to be 83 ± 2.5 (SE) atm, and all the other gases elevated this threshold significantly at doses which were well below those required to cause anesthesia at these pressures. In control experiments without anesthetic, 17% of the animals exhibited the more severe symptoms of the high pressure neurological syndrome, such as clonic and tonic convulsions, without proceeding through a complete spasm phase. This partly reflects the small pressure increments occurring between successive phases of the syndrome; for example, the ED_{50}'s for clonic and tonic convulsions were 88 ± 1.4 and 96 ± 3.4 atm. The proportion of animals with complete spasms is indicated in Table 2.

Our data for clonic convulsions agree well with a value of 89 atm reported by Brauer (24) for CD-1 mice compressed at 60 atm per hour. This threshold pressure is somewhat dependent on compression rate, varying from 103 atm at 10 atm hr^{-1} to 86 atm at 100 atm hr^{-1} (24). In our work, therefore, the compression rate was kept constant within ±2%. Only one complete spasm threshold has been reported in CD-1 mice: this value of 87 atm was obtained at a compression rate of 60 atm hr^{-1} (17).

TABLE 2

Median pressures for the appearance of complete body spasms in mice breathing helium-oxygen and helium-oxygen-anesthetic gas mixtures

Anesthetic gas	ED_{50} ± SE	Scale parameter ± SE	No. responding/total no.
	atm		
None	83 ± 2.5	12 ± 3.5	33/40
0.1 atm N_2O	90 ± 6.3	11 ± 7.5	9/10
0.2 atm N_2O	103 ± 8.3	8 ± 8.0	6/10
0.4 atm N_2O	99 ± 2.0	9 ± 6.9	9/10
0.8 atm N_2O	100 ± 1.5	23 ± 18.0	10/10
1.5 atm N_2O	120 ± 1.3	13 ± 8.4	10/10
3 atm N_2	88 ± 4.0	17 ± 16.0	8/10
15 atm N_2	110 ± 3.0	30 ± 21.0	10/10
30 atm N_2	112 ± 6.6	13 ± 9.4	10/10
45 atm N_2	120 ± 5.1	20 ± 13.0	9/9
3 atm Ar	95 ± 4.0	20 ± 12.0	10/10
8 atm Ar	108 ± 6.9	12 ± 9.1	8/10
18 atm Ar	129 ± 3.6	29 ± 23.0	10/10
3 atm CF_4	86 ± 3.3	21 ± 16.0	10/10
10 atm CF_4	88 ± 3.8	19 ± 13.0	10/10
17.2 atm CF_4	137 ± 10.6	10 ± 8.5	7/10
25 atm CF_4	115 ± 6.2	17 ± 18.0	6/10
0.2 atm SF_6	91 ± 6.5	11 ± 9.1	7/10
0.4 atm SF_6	89 ± 6.7	10 ± 7.8	9/10
1.0 atm SF_6	83 ± 9.9	9 ± 7.2	10/10
2.5 atm SF_6	87 ± 8.5	8 ± 5.2	10/10

Theoretical Analysis of Data

It is not possible to account fully for pressure reversal of anesthesia by addition to either the Meyer-Overton (1) or Mullins (2) hypothesis of a term which takes into account the dependence of the lipid solubility of the anesthetic on pressure (5). The original Mullins formulation was stated in terms of volume occlusion of free space in membranes. Although such a mechanism is now known to occur in a number of cases of cytoplasmic protein-anesthetic interactions (25, 26), it is not clear how it can account for the pressure reversal of anesthesia (2). If the formulation is restated in terms of the membrane expansion which has been shown more recently (27–29) to be caused by small hydrophobic molecules, then pressure reversal is readily accounted for by adding a term for the isothermal compressibility (β) of the membrane (5). This critical volume hypothesis of anesthetic action, which we have defined previously (5), may be written down in its most general form as

$$\Delta V_c = \sum_{i=1}^{n} \Delta V_i - \beta P_T \qquad (1)$$

Where ΔV_c is the volume change which is associated with anesthesia (or any other end point), ΔV_i is the expansion caused when the ith anesthetic dissolves in the hydrophobic region and P_T is the total mechanical pressure.

Equation (1) may also be used to formulate the critical volume hypothesis applied to the high pressure neurological syndrome (11). In our case ΔV_c will be the volume change at which complete spasms are observed. Since these may be produced by hydraulic compression of mice in oxygenated fluorinated hydrocarbon solvents (13, 14) (when $\Delta V_i = 0$), it is evident that this syndrome is accompanied by a negative volume change since under these conditions equation (1) reduces to

$$\Delta V_c = -\beta P_T \qquad (2)$$

Since all gases, including helium, contribute positively to ΔV_i, it follows that for helium the term $(\Delta V_{He} - \beta P_{He})$ must be less than zero both at the anesthetic site and at the site mediating complete spasms. If this were not so the model would predict helium to be anesthetic and the high pressure neurological syndrome would not be observed in helium-oxygen atmospheres.

In order to perform a quantitative test of these two versions of the critical volume hypothesis it is necessary to know the physical parameters of the sites of action which are required to solve equation (1) in each case. Since the sites of action are not identified this is not possible, but it is well known that certain apolar solvents provide good models of the anesthetic site. It is possible that such solvents might also provide a suitable model for the site associated with complete spasms (11).

Choice of Solvent Models for Anesthesia and Complete Spasms

It is possible to choose objectively the best solvent model of a site of action of inert gases if pharmacological data are available for some fully fluorinated gases (10, 19, 20). This procedure is based on the observation that when a set of nonpolar solvents of graded solvent power are compared it is found that fully fluorinated gases deviate systematically, depending on solvent power, from the correlations always observed for the nonfluorinated gases. By minimizing these deviations the solvent power of the site of action may be defined objectively.

Hildebrand (30) defined the solvent power of a solvent in terms of a solubility parameter, δ, which is defined as

$$\delta^2 = \frac{\Delta E_v}{V_m} \qquad (3)$$

where ΔE_v is the heat of vaporization of the solvent at constant volume and V_m is its molar volume. Strictly speaking, equation (3) applies only to nonpolar solvents and to solutes and solvents of equal molecular size. However, in practice semiquantitative agreement is found over a wider range of situations (30). Sufficient solubility data were available to carry out such calculations for six solvents, including water which was not included in the final analysis because of its high polarity. The analysis of the deviations of the fluorocarbons, which is described below, differs somewhat from that used before (20) since we wished to apply it to our data obtained at all pressures. For each ED_{50} at a given pressure (P_T) in Tables 1 and 2, ΔV_i may be calculated for each gas at a partial pressure, P_i, in each solvent since (5)

$$\Delta V_i = \frac{\bar{V}_i X_i P_i}{V_m} \qquad (4)$$

where, $\bar{V}_i$ is the partial molar volume of the gas in the solvent, X_i is its mole fraction solubility at a partial pressure of one atmosphere, and V_m is the molar volume of the solvent. Corrections must be made to equation (4) to account for the non-ideal behavior of gases and for the dependence of X_i upon total pressure (5). These corrections reduced the value of ΔV_i calculated by equation (4). In general such corrections lay in the range of 10–20%, but in a few cases are in the range 50–60%. Sources of the physical parameters used in these calculations are given in the appendix.

$\Sigma\Delta V_i$ in equation (1) was calculated for each datum in Tables 1 and 2 and plotted against P_T (equation 2). A solvent which ideally represented one of the sites of action should yield a linear plot with a slope of β, the compressibility, and an intercept of ΔV_c. Each gas gave such a line; those for nitrous oxide, nitrogen and argon were essentially co-linear in all solvents, while those for sulfur hexafluoride and carbon tetrafluoride were above the other gases at low solubility

parameter and below at high solubility parameter (Fig. 1). Hexafluoroethane was not included in this analysis because there are

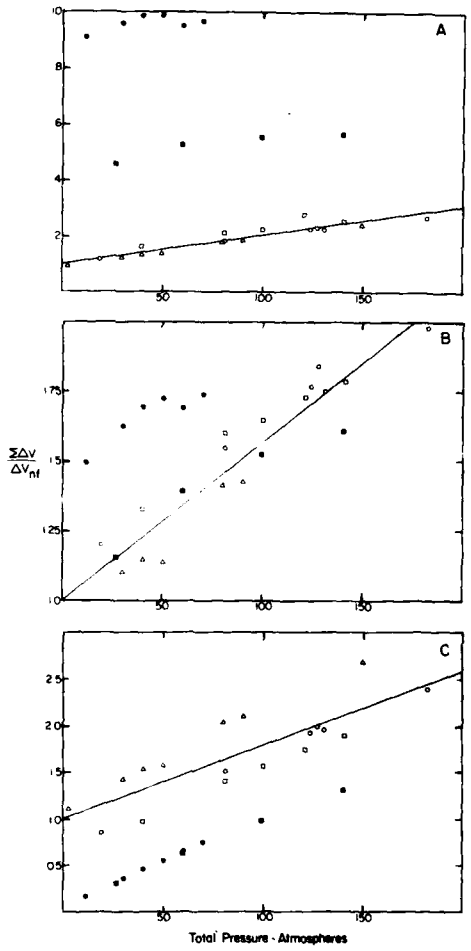

FIG. 1. *Plots of the calculated expansion caused by ED_{50} doses of anesthetics with helium ($\Sigma\Delta V$) at various pressures*

The expansion has been normalized to the intercept for the inert gases, (ΔV_{nf}), for comparative purposes. Key: N_2 □; Ar ○; N_2O △; CF_4 ■ and SF_6 ●. For the fluorinated solvents (A) $\delta = 6$, for carbon disulfide (B) $\delta = 10.0$ and for water (C) $\delta \simeq 23$. Note that the ordinates of each figure are plotted with a different scale. Values of $(\Delta \bar{V}_f - \bar{V}_{nf})/|\Delta \bar{V}_f + \Delta \bar{V}_{nf}|$ (see text) in each solvent for anesthesia and complete spasms respectively were: fluorinated solvents 0.75 and 0.76; cyclohexane ($\delta = 8.2$) 0.39 and 0.09; benzene ($\delta = 9.2$) 0.24 and 0.05; carbon disulfide 0.08 and 0.01 and octanol ($\delta = 10.3$) 0.10 and 0.02.

insufficient solubility data. To quantify these deviations of the fluorinated gases a straight line was fitted through the nonfluorinated gases to yield an intercept ($\Delta \bar{V}_{nf}$). A parallel line was fitted to the data for the fluorinated gases to yield a second intercept ($\Delta \bar{V}_f$). For each solvent the expression $(\Delta \bar{V}_f - \Delta \bar{V}_{nf})/|\Delta \bar{V}_f + \Delta \bar{V}_{nf}|$ was plotted against the solubility parameter, δ. Linear regression yielded zero deviation at δ (anesthesia) = 10.8 ± 0.15 (SD) and δ (complete spasm) = 9.8 ± 0.40 ($p = 0.001$) $(Cal/cm^3)^{1/2}$. The complete spasms site is thus a slightly better solvent (lower δ) than the anesthetic site. However, such small differences in solubility parameter should be regarded with reservation because the analysis is critically dependent on the fluorinated gases with which the experimental difficulties are greatest. Thus a previous analysis with poorer data for carbon tetrafluoride yielded δ (anesthesia) = 10 (20).

Results of the Critical Volume Hypothesis Analysis

The actual solvents which most closely represent the solubility parameters defined above are octanol ($\delta = 10.3$) and carbon disulfide ($\delta = 10.0$). We have chosen the latter for detailed consideration since the requisite physical properties have been better defined. We have also considered olive oil both because of its historical importance and because its molar volume is closer to that of membrane components such as phospholipids. The absolute values of ΔV_c given by it should thus be more realistic than those given by carbon disulfide. The results obtained by calculating $\Sigma\Delta V_i$ (equation (4)) for all the data in Tables 1 and 2 and analyzing them according to equation (1) are given in Table 3 and in Fig. 2. The parameters obtained for the two sites of action are not grossly dependent on the solvent model, although as expected the volume changes predicted by olive oil are less marked than those predicted by carbon disulfide. In fact the success of olive oil at modeling these sites of action would seem to vindicate a long tradition.

Two major conclusions stand out. First, increases or decreases in volume or density in hydrophobic sites in the central nervous system may lead to profound effects, such

TABLE 3

Results of calculations to test the critical volume hypothesis

Model solvent	Effect	Critical volume change (%) ± SD	Compressibility ($\times 10^{-4}$ atm^{-1}) ± SD	Correlation coefficient
Carbon disulfide	anesthesia	+0.63 ± 0.036	2.1 ± 0.39	0.72
	complete spasms	−0.6 ± 0.17	11 ± 1.6	0.83
Olive oil	anesthesia	+0.43 ± 0.029	2.4 ± 0.32	0.83
	complete spasms	−0.5 ± 0.12	8.4 ± 0.12	0.85

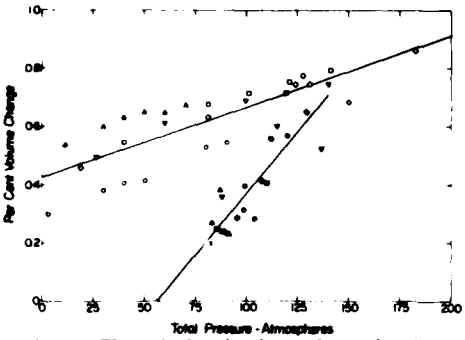

FIG. 2. *The calculated volume changed at ED_{50}s for anesthesia (open symbols) and pressure-induced spasms (closed symbols) using olive oil as a model solvent*

Key: N_2 □, ■; Ar ◇, ◆; N_2O ○, ●; CF_4 ▽, ▼; SF_6 △, ▲ and helium ×.

as the depression and excitation studied here. The magnitude of these changes seems to be in the range of ½–1% although this magnitude reflects somewhat the choice of the model to represent the hydrophobic site. Second, more than one site of action is involved in the two phenomena studied here, the site mediating complete spasms being four to six times more compressible than that mediating anesthesia. These conclusions confirm a preliminary test of these two applications of the critical volume hypothesis which was based on scant data derived from heterogeneous sources (11). A difference in the pressure sensitivity of anesthesia and convulsions has also been noted when barbiturates are used (31).

A number of interesting corollaries follow from these calculations. Equation (2) predicts that mice compressed hydraulically would experience complete spasms at 56 atm (olive oil model), a prediction which could be checked experimentally. For tonic convulsions this threshold is found to be 62 atm (14).

Figure 2 illustrates clearly that subanesthetic doses are required initially to prevent complete spasms. However, since the compressibility of the spasm site is greater, then as the pressure is progressively raised, relatively higher doses of inert gas are required to prevent spasms until above 148 atm more than an anesthetic dose is required. In fact two reports of convulsing anesthetized mice at extreme pressures have been published (31, 32).

An important point is that the theory does not predict that helium will compress all hydrophobic sites. Equations (1) and (4) show that a negative volume change only occurs when $(V_{He} \cdot X_{He}/V_m) < \beta$. If, for example, X_{He} is larger (i.e., in a solvent of lower δ) net expansion might occur. Thus, synergism between helium and an anesthetic cannot be taken as *prima facie* evidence that a mechanism inconsistent with the critical volume hypothesis is involved, unless quantitative studies are performed to derive δ and β or hydraulic pressure is used as an added criterion. Synergism between helium and nitrogen has been reported in some, but not all, behavioral tests in rats (33), while synergism between hydraulic pressure and anesthetics has occasionally been noted (34).

Similarly, if we consider gases just a little more soluble than helium at the anesthetic site of action, we find that neon, which is 1.2 times more soluble than helium, causes a volume change at 100 atm partial pressure of only 0.035% (olive oil model). This is consistent with the finding that high pressures of neon do not change the potency of nitrous oxide (21).

It is also possible to make an estimate from this data of the thermal expansion

coefficients, α, of the sites of action. For a simple nonpolar solvent one may write (30, 35)

$$\delta^2 = \left(\frac{\partial E}{\partial V}\right)_T = T\left(\frac{\partial P}{\partial T}\right)_V \simeq T\frac{\alpha}{\beta} \quad (5)$$

Using the parameters in Table 3 for carbon disulfide, equation (5) yields an α of 8×10^{-4} for the anesthetic site and 4×10^{-3} $°C^{-1}$ for the spasm site. The actual α coefficient for carbon disulfide itself is 1.2×10^{-3}, and for oleic acid is 9.6×10^{-4} (36), so these predictions are not unreasonable in spite of the assumptions inherent in their derivation.

DISCUSSION

The success of the critical volume hypothesis in providing a unified description of the pressure reversal of anesthesia and of the amelioration of the high pressure neurological syndrome by inert gases is remarkable. When data for the fluorinated gases are available so that an objective choice of model solvent may be made, the only remaining adjustable parameter is the compressibility of each site of action. Physically realistic values of this parameter must lie within a fairly narrow range of values spanning little more than an order of magnitude. Thus, the actual values for olive oil and for carbon disulfide are 6 and 7×10^{-5} atm^{-1}, respectively. For hexadecane and pentane the values are 7 and 17×10^{-5} atm^{-1} (36). On this basis the values of compressibility found in Table 3 are seen to be quite plausible.

A putative site of action for anesthetics is the lipid bilayer region of membranes. In principle we should be able to test this model, but the data are currently inadequate. Some data (37, 38) suggest that gas solubility in olive oil is less than twice that in bilayers. Thus we would expect lipid bilayers to yield slightly smaller critical volumes and compressibilities than those shown for olive oil in Table 3. The only available bilayer compressibility is for the liquid crystalline phase of dipalmitoyl lecithin and it is 10^{-4} atm^{-1} (39). Thus lipid bilayers will probably provide about as good a model as the simple solvents used here. Moreover the anisotropy of bilayers may need to be considered (5).

Although anesthetics are relatively nonspecific when compared to receptor directed drugs, they do in fact exhibit considerable selectivity at low doses when studied in well defined systems (40–42). Attempts have been made to explain some of this specificity on the grounds of differential solubility in target membranes (2, 43). This is not the case in our study where the two sites have barely distinguishable solubility properties. The spasm site is most clearly differentiated from the anesthetic site by its greater sensitivity to pressure, which results from a fivefold higher compressibility. Thus we have characterized two separate sites of action which reach a critical point and cause a physiological response, one at a positive volume change and the other at a negative volume change. Under ambient pressure conditions both will be almost equally expanded by an anesthetic but whether such expansion would prove critical to the HPNS site, and if so what the result would be, cannot be defined. It would seem quite reasonable to suppose, however, that a sufficient volume change in any direction could have profound effects (44). Only studies in simpler systems will answer such questions.

The demonstration of two distinct sites in the central nervous system where anesthetics and pressure interact antagonistically raises questions about the simplifying assumptions in our treatment of the critical volume hypothesis. We have assumed that the anesthetic-pressure interaction which mediates a given physiological response, such as righting reflex, occurs at a single class of site which has uniform physical properties. Thus, even when the assumption of direct pressure-anesthetic interaction is retained, our results might merely reflect the average physical properties of a number of distinct sites, all of which are sensitive to both anesthetics and pressure. In isolated ganglia (42) the lowest effective doses of anesthetic block only one pathway, but at higher doses others are blocked. In the central nervous system a number of distinct pathways might be blocked over a very narrow dose range and our idea of

distinct sites might then need to be replaced by a continuum of sites. Our results cannot fully resolve this problem, but the linearity observed in each case in Fig. 3 does suggest that for a given physiological response there are no strong interactions between two or more sites with different physical properties. Furthermore the anti-HPNS and the anesthetic sites are distinct and independent, since when both have supra-critical volume changes (e.g., above 150 atm, see Fig. 3) we observe signs of the HPNS in anesthetized animals (31, 32).

With the above reservations the critical volume hypothesis provides a description of the thermodynamic properties of a hydrophobic region at which anesthetics appear to act. To proceed further requires thermodynamic measurements of the properties of actual putative sites. At least three mechanisms can be distinguished. The anesthetic might interact directly with hydrophobic regions in an excitable protein (27), or it might affect such a protein by a perturbation of its lipid environment, either by expanding and thereby fluidizing it (45) or by changing the phase relationships in it (46). These three models are qualitatively consistent with the predictions of the critical volume hypothesis. To distinguish between them with quantitative calculations requires thermodynamic measurements that are not yet available for gaseous anesthetics, although the solubility parameter of erythrocytes is close to those predicted here (47). Even when such data are available a successful model can only be shown to have properties consistent with those of the site of action. It then provides a powerful predictive and rather exclusive thermodynamic framework without specifying the details of mechanisms. The deductive elucidation of the latter requires a different approach.

APPENDIX

Physical quantities used in the calculations were generally available in the literature. Solvent molar volumes, solubility parameters and gas solubilities were taken from a number of useful reviews (29, 30, 48–51). Partial molar volumes of gases in solvents are not all available, but since they vary little with solvent we used the values for benzene in all cases. Sources have been given in a previous publication (5), except the values for argon (44.6 ml/mole) and hexafluoroethane (110 ml/mole) which were taken from references 52 and 53. Virial coefficients for pure gases and for gas mixtures have also been given previously (5) except for B (Ar-He) for which +24 ml/mole was estimated (54).

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MOLECULAR PHARMACOLOGY, 15, 729–738

On the Coupling between Anesthetic Induced Membrane Fluidization and Cation Permeability in Lipid Vesicles

KAM-YEE PANG, TIEN-LAN CHANG AND KEITH W. MILLER[1]

Departments of Pharmacology and Anaesthesia, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114

(Received July 17, 1978)
(Accepted November 16, 1978)

SUMMARY

PANG, KAM-YEE, CHANG, T-L. & MILLER, K. W. (1979) On the coupling between anesthetic induced membrane fluidization and cation permeability in lipid vesicles. *Mol. Pharmacol.*, **15**, 729–738.

The effect of anesthetics on the rubidium-86 ion efflux from phospholipid vesicles was studied in the presence of the ionophores gramicidin A and valinomycin and in the absence of ionophores. Anesthetics of known lipid/buffer partition coefficient were used. Pentobarbital, halothane and butanol all increased the three types of ion efflux. The effects were linearly dependent on each anesthetics' membrane concentration over a range of anesthetic mole fractions in lipid from 0.02 to 0.3, and concentrations known to produce anesthesia produced clearly significant increases in each case. The effect of a given membrane concentration of all the anesthetics on each of the three modes of ion efflux was similar, suggesting that a single perturbation of the lipids is involved in every case. The effects of anesthetics on cation permeability correlated better with their perturbation of lipid bilayers (reported by a freely rotating hydrophobic fluorescent probe [1,6-diphenyl 1,3,5-hexatriene]) than with those reported by spin-labeled lipid probes undergoing anisotropic motion. Coupling between the permeability increases and the perturbation of the bilayer structure was strong, the functional changes being about an order of magnitude larger than the structural changes. The lipid perturbation hypothesis of anesthetic action has been criticized because the structural perturbations observed at anesthetic concentrations are small, but such strong coupling between structural and functional changes may resolve this problem.

INTRODUCTION

The site of action of anesthetics has received considerable attention recently. Because the physiological site (or sites) of action within the central nervous system remains undetermined, much of this work follows the classical physicochemical approach of correlating anesthetic potency with the physical properties of anesthetics. Advances in basic membrane science have provided new models and techniques that have considerably extended the scope of such an approach. The possibility that anesthetics interact directly with hydrophobic regions of membrane proteins remains untested for lack of a suitable model, while considerable progress has been made in understanding interactions between anesthetics and lipid bilayers, which present a readily available, well defined model.

This research was supported by an Office of Naval Research Contract (N00014-75-C-0727) with funds provided by the Naval Medical Research and Development Command.

[1] Research Career Development Awardee of NIGMS (GM00199).

0026-895X/79/030729-10$02.00/0

Changes in bilayer fluidity and permeability provide successful models for the major features of anesthetic potency (1–3) and its reversal by pressure (4–6) but a number of problems remain unresolved. First, there is some question whether fluidity changes do in fact occur at low concentrations of anesthetic (7). Even though these doubts have themselves been questioned by more recent work on halothane (8), the size of the perturbations measured are at least comparable to the errors involved in spectroscopic studies of structural perturbations. But then, the volume changes predicted by theoretical studies of pressure reversal are also small (9). Second, the problem of how a perturbation of the state of the lipid bilayer may be coupled to and disrupt the function of some excitable protein remains largely unstudied. A small change in the state of lipids may cause a large change in a protein's functional capacity. If so this would resolve the difficulty associated with the small changes observed in lipid bilayer structure. A third problem, that of specificity (i.e., how does a generalized lipid perturbation have fairly specific effects on membrane function), may be related to specificity either in the primary anesthetic-lipid interaction (10) or in the coupling of the lipid perturbation to protein function. A recent study that examined the effect of benzyl alcohol in the lipids of a calcium-magnesium ATPase from sarcoplasmic reticulum sheds some light on this complex problem but high concentrations of this agent were required (11), and electrophysiological evidence suggests excitable ionophores would be more suitable models of anesthetic action (12, 13).

One approach to the problem of lipid-protein coupling is to study in well defined lipid bilayers the function of simple ionophores of known structure and defined mechanism, for example certain antibiotics. Since these systems are often more sensitive to anesthetic perturbations than are spectroscopic probes of the state of lipid bilayers, they provide incidently an approach to the determination of the size and linearity of the lipid perturbation at low anesthetic concentration.

Previously it has been shown that the ion carrier, valinomycin, provides a useful model for examining anesthetic (1) and pressure (4, 5) induced changes in the lipid bilayer. However, excitable fluxes in nerves are carried by ion channels, and the observation that the life time of gramicidin A channels in black lipid membranes is related to membrane thickness (14) suggested that it might be a more suitable model. Recently several authors have elaborated this possibility in some detail (15–17). We chose to study gramicidin A in lipid vesicles both because the primary anesthetic-lipid interaction has been well characterized in them and because black lipid membranes, which would offer the opportunity of resolving single channel events, are saturated with solvents such as hexadecane. We argued these would influence the subtle anesthetic-lipid interaction and it seemed wiser, at least initially, to characterize the effects of anesthetics in the absence of such potentially confounding variables.

We present here a comparison of the effects of anesthetics on ion fluxes mediated by the carrier, valinomycin, and the channel former, gramicidin A, in phospholipid bilayers, using a new method of higher precision than previously reported. Experiments were carried out under conditions for which the membrane concentration of the anesthetic can be accurately estimated. The magnitude of the observed effects can, thus, be readily related to the anesthetic concentration.

MATERIALS AND METHODS

Valinomycin (A grade) was obtained from Calbiochem, Los Angeles, Cal., gramicidin A from Nutritional Biochemicals Corp., Cleveland, Ohio, egg phosphatidylcholine and phosphatidic acid (Grade 1) from Lipid Products, Surrey, England, and cholesterol (chromatographic grade) from Sigma Chemical Company. All were used as supplied, except cholesterol which was recrystallized from methanol. Rubidium-86 and calcium-45 were from New England Nuclear, Boston, Mass. 1,6-Diphenyl-1,3,5-hexatriene (DPH) and tetrahydrofuran were from Aldrich, Milwaukee, Wis. Spin-labeled 8-doxylstearic acid was synthesized by Dr. M. J. Pringle in this laboratory. All

other reagents were analytical grade.

Phospholipid vesicles were prepared by first drying down 90 μmoles of phospholipid from stock solutions in chloroform containing 96 mole % phosphatidylcholine and 4 mole % phosphatidic acid. Five milliliters of buffer (0.12 M KCl, 0.03 M RbCl (containing 0.25 mCi of ^{86}Rb), Tris-HCl 10 mM at pH 7.0 or 8.1) was added and the suspension sonicated in a stoppered conical tube in a bath sonicator (Heat Systems, model 5 × 5) at 25° under nitrogen. The clear suspension was passed through a Sephadex G-50 (coarse) column, preequilibrated with nonradioactive buffer, the vesicles collected in the void volume, and diluted to 0.2–0.4 mM phospholipid in the above buffer without radiolabel and containing in some cases anesthetic. After 15–30 min equilibration in a water bath at 25°, valinomycin, or gramicidin A, was added in ethanol to give, usually, a final concentration of about 1–10 nM respectively. This was sufficient to ensure a flux rate 6–10 times higher than the background due to passive permeability. The final ethanol concentration, 0.01% v/v, was shown in control experiments to have a negligible effect.

Ion leak rate from the vesicles was determined by ultrafiltration. At intervals of 10–20 min 2 ml. aliquots were tranfered to 10 ml filtration cells (Amicon, Lexington, Mass., model 12) and filtered through an XM-50 ultrafilter (Amicon) under 4 atm of nitrogen. The first 0.3 ml of filtrate was discarded and the remainder (usually 1.2 ml) was collected for scintillation counting in aquafluor (New England Nuclear, Boston, Mass.). A few samples were analyzed by Cherenkov radiation. Filtration time varied from 1½ to not more than 5 min. We have previously shown that no vesicles appear in the filtrate (19). The incubation mixture was also assayed for phosphorous (20) and total radioactivity.

In some experiments both rubidium and calcium fluxes were determined simultaneously and the buffers then contained 0.09 M RbCl and 0.03 M $CaCl_2$ in 10 mM Tris-HCl at pH 7.

Because of its volatility halothane incubations were performed in a 30 ml glass syringe capped by a two-way valve through which appropriate amounts of saturated halothane solution were added. Final halothane concentrations were determined by gas chromatography at the end of each experiment. Even with these precautions concentrations were often 5–20% below those expected.

Permeability was treated as a first order process (1, 5) given by

$$\frac{dn}{dt} = \frac{PA}{V}(N - n) \quad (1)$$

where n is the number of counts per minute at time t which have leaked from liposomes of surface area A and internal volume V, containing N counts per minute. P is the permeability coefficient. Plots of ln $(N - n)$ versus t were found to be linear over 300 to 480 minutes, depending on the concentration of ionophores used, and the slope is thus proportional to P.

Samples for fluorescence depolarization were prepared by the method of Lenz et al. (21). Phosphatidylcholine suspensions were sonicated with the microtip of a probe sonifier (Heat Systems, model W185) at 0°. Metal debris and large vesicles were precipitated at 100,000 × g for 30 min. Pentobarbital was mixed with lipids before sonication, while halothane was added after centrifugation, using the technique described above. Four and a half milliliters of liposome suspension (about 0.7 mM) was mixed with 1 μl of 2 mM DPH in tetrahydrofuran and vigorously stirred for 1 hr. Fluorescence depolarization was determined at 25° in a subnanosecond spectrofluorimeter (S.L.M. Series 400, Urbana, Ill.). The sample was excited at 360 nm and recorded at 430 nm. Microviscosity was calculated from the fluorescence depolarization by the method of Shinitzky and Barenholz (22). The depolarizations reported at 10, and at 30 MHz modulation were not significantly different.

Samples of phospholipid vesicles with anesthetics for spin label studies were prepared according to the method described previously (23).

RESULTS

The diffusion of $^{86}Rb^+$ ion through the lipid bilayer was treated as described above

(equation 1 and figure 1). If values for the internal volume and surface area are assumed the permeability coefficient, P, may be calculated. The internal volume of a 4% phosphatidic acid–96% phosphatidylcholine liposome has been shown to be 6.7×10^{-18} cm^3 and its external surface area to be 8.6×10^{-12} cm^2 (24). Hence, for $^{86}Rb^+$ passive diffusion at 25° we calculated $P = 4.8 \times 10^{-13}$ cm/sec compared to a value for $^{42}K^+$ of 7.3×10^{-13} cm/sec. at 37° in a similar preparation (1) and of 1.3×10^{-14} cm/sec at 4° in egg phosphatidylcholine vesicles using slightly different numbers for external surface area and internal volume (25).

The carrier mechanism of valinomycin transportation has been well established both in black lipid membranes and in liposomes (26–30). Although the evidence that gramicidin A dimerizes to form ion conducting channels in black lipid membranes is strong, its effects in vesicles have not been characterized (29, 31). Indeed the inability to record single channel events in vesicles makes a full characterization difficult. Nonetheless, the presence of hydrocarbon solvent in black lipid membranes renders them needlessly complex for anesthetic studies. We therefore characterized the gramicidin A mediated cation flux in our vesicles as follows. First, incorporation of gramicidin A into vesicles caused the same increase in efflux when the antibiotic was added to the suspension or when it was dried down with the lipid before the vesicles were prepared. This reduced the probability that the increase in efflux resulted from some nonspecific interaction. Secondly, gramicidin selected rubidium over calcium (Fig. 1) just as it does in black lipid membranes (29). Valinomycin showed the same selectivity. We were unable to demonstrate a dependence of permeability on the square of the gramicidin concentration because the range of permeabilities that can be studied by our technique is too narrow. However, permeability increased with gramicidin concentration over the limited range studied. In a typical experiment the mean number of gramicidins per liposome was 0.4. Assuming that all gramicidin is present as dimers (31), up to 20% of the liposomes

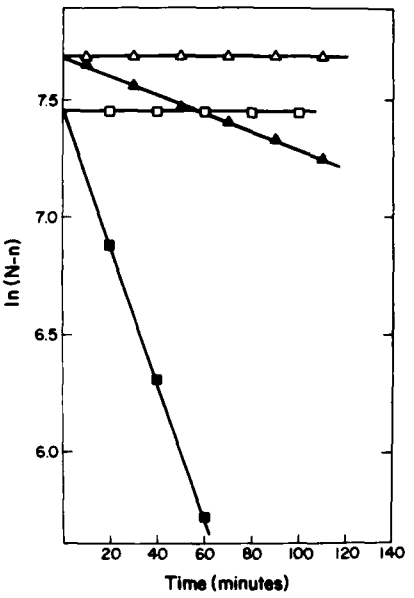

FIG. 1. *The efflux of $^{86}Rb^+$ (solid symbols) and $^{45}Ca^{2+}$ (unfilled symbols) from 96% egg phosphatidylcholine-4% egg phosphatidic acid sonicated vesicles*

Triangles, in the presence of 0.1 μM gramicidin A and 0.59 mM phospholipid; squares in the presence of 1 nM valinomycin and 0.57 mM phospholipid. N is the total counts and n the filtrate counts determined at time t. The slopes of the lines through the points for rubidium and calcium efflux are, respectively, $-3.95 \pm 0.13 \times 10^{-3}$ (SD) and $-1.7 \pm 2.2 \times 10^{-5}$ in the gramicidin experiment, and $-2.89 \pm 0.036 \times 10^{-2}$ and $-9.5 \pm 2.8 \times 10^{-5}$ in the valinomycin experiment and $-2.3 \pm 0.12 \times 10^{-4}$ and $-0.5 \pm 5.8 \times 10^{-6}$ in the unmodified liposomes (not shown).

contain a channel at any given time. Under these conditions the measured cation flux is 2×10^{-3}/min and remains linear for at least 300 min. This suggests that gramicidin, like valinomycin, must be in dynamic equilibrium with the liposomes on the time scale of this experiment. Studies in planar bilayers suggest a single channel might empty a vesicle in much less than one second, but no evidence for a rapid efflux phase from those vesicles initially containing two gramicidins is seen at 0.1 μM (Fig. 1), or at 1 μM gramicidin (not shown). Possibly the kinetics of channel formation is strongly affected by vesicle curvature, but in any event the rate-limiting process in the fluxes

measured here remains uncertain.

All three anesthetics increased all the ionic fluxes examined in a concentration dependent manner. Control experiments showed that pentobarbital at high pH had little effect on the valinomycin mediated flux. The effect increased at lower pH showing the uncharged molecule to be the primary affector. The pH dependence of pentobarbital partitioning has been determined in egg phosphatidylcholine (19). It is thus possible to calculate the dependence of the permeability increase upon the bilayer concentration of pentobarbital. Figure 2 shows the data of several experiments for each of the cation fluxes. The effect of the anesthetic on all three types of permeability is remarkably consistent and independent of pH when the membrane concentration is adjusted appropriately. Halothane yielded similar results over the same concentration range (not shown) when a partition coefficient of 152 moles halothane per ml lipid/ moles halothane per ml buffer (Porter & Miller, unpublished data) was used to calculate the bilayer concentration. For butanol a partition coefficient of 3.2 was assumed. This value is for dimyristylphosphatidylcholine (32), but comparison of results obtained for benzyl alcohol and for butane, both in saturated and unsaturated lipids, suggests that the value used will not be in error by more than 30% (33, 34). We thus calculate that the valinomycin mediated cation flux in the presence of butanol was linear down to a mole fraction of 0.02 in the lipid (Fig. 3). The slopes of these graphs quantitate the dependence of the increase in permeability on membrane concentration. The results of a linear regression through the origin in each case are given in Table 1. When the best fit was not constrained to pass through the origin the predicted intercept varied randomly above and below $\ln (P/P_0) = 0$. The mean of all intercepts determined was −0.025, and in no case was the intercept more than two standard deviations from the origin. (In five of seven cases it was within one standard deviation of the origin.) The mean correlation coefficient was 0.983. The linearity of these plots is thus established within quite

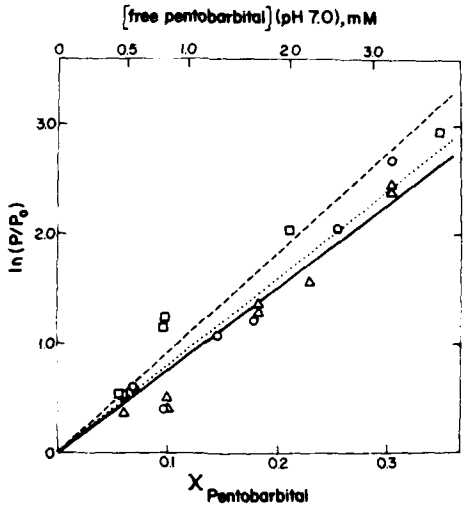

FIG. 2. *The effect of pentobarbital on rubidium efflux in the absence of ionophore, ○, and in the presence of valinomycin, □, and gramicidin A, △*

The membrane concentration was calculated as described in the text. The ratio of anesthetic permeability, P, to control permeability, P_o, is determined from the slopes of plots such as those in Figure 1. Least squares fitted lines, through the origin for no ionophore (dotted); valinomycin (dashed); and gramicidin A (solid).

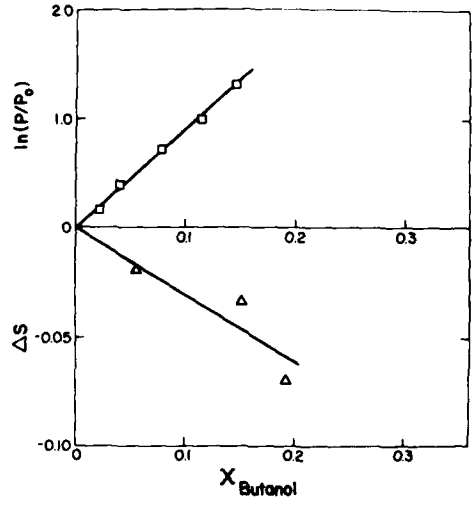

FIG. 3. *The effect of n-butanol on valinomycin mediated rubidium permeability □, and on the change in order parameter, ΔS, of 8-doxylstearic acid ▽*

close limits. Comparison of our data for valinomycin with a previous determination using a dialysis technique (5) shows good agreement for pentobarbital and butanol, although our errors are considerably smaller. Our value for halothane is larger than the previous one, emphasizing the need to directly monitor the concentration of this volatile agent.

Table 1 shows that when these anesthetics are examined at constant membrane concentration, the effect of each of them on a given type of cation flux is remarkably similar.

Figure 3 also shows the dependence of the order parameter of 8-doxylstearic acid in phospholipid vesicles on the membrane concentration of butanol. Order decreases in an approximately linear fashion, but the errors in determining ΔS are relatively larger at a given membrane concentration than those of the permeability study.

The fluorescence depolarization data for DPH showed that both halothane and pentobarbital (pH 7.0) fluidized the bilayer. The calculated microviscosity of the control bilayers was 0.96 poise, in agreement with Shinitzky and Barenholz (22). At mole fractions of 0.05 pentobarbital and 0.38 halothane the microviscosity was 0.87 and 0.63 poise respectively. Under these conditions fluorescence life times detected by phase and modulation methods were homogeneous, but we were unable to study higher concentrations of pentobarbital. Control experiments showed that pentobarbital at a mole fraction of 0.5 reduced the quantum yield of steady state DPH fluorescence, and in the presence of vesicles it showed different fluorescence life times by phase and modulation detection methods. Recent studies have suggested that DPH does not rotate freely in bilayers of saturated lipids and that the assumptions inherent in microviscosity calculations may be invalid (35, 36). However, 20–30° above the phase transition there is free rotation (36), so that our use of the microviscosity formalism in egg lethicin bilayers is probably a good approximation.

TABLE 1

The increase in $^{86}Rb^+$ efflux in 96% egg phosphatidylcholine:4% phosphatidic acid liposomes in the presence of anesthetics

The figures are the slopes of plots such as Figure 2. The units are ln (P/P_0) at unit mole fraction of anesthetic in lipid ± S.D.

Ionophore	Anesthetic		
	Pentobarbital	Halothane	*n*-Butanol
None	8.0 ± 0.43	10.4 ± 0.45	—
Valinomycin	9.2 ± 0.48	9.0 ± 0.29	9.1 ± 0.12
Gramicidin A	7.5 ± 0.32	6.8 ± 0.30	(5.7)[a]

[a] Single determination.

DISCUSSION

Our results establish with high accuracy the relationship between the concentration of the anesthetic in the bilayer and the ion permeability through it. They enable us to estimate the size of the change at physiologically relevant concentrations and to examine the coupling between the lipid perturbation and the function of carrier and pore forming ionophores. These experiments were not designed to directly evaluate the mechanisms involved; nonetheless it is possible to relate these results to more mechanistic studies and to correlate them with structural changes induced in the bilayer.

The dependence of the anesthetic induced increase in ion flux upon membrane concentration was in each case highly linear. No deviations were noted even though the anesthetic mole fraction was varied over more than an order of magnitude. Two conclusions relevant to anesthetic studies may be inferred. First, in these permeability studies it is valid to extrapolate from high ($\times \sim 0.35$) to low anesthetic concentrations, which suggests that the same will be true of measurements, often made with less sensitive techniques, of the underlying lipid perturbation. (This might not be so in other types of permeability if the coupling between lipid perturbation and ionophore is nonlinear). Second, the suggestion that there is a nonlinear relationship between anesthetic concentration and membrane fluidization, particularly at low concentrations (7) appears now to be highly improbable. Our finding in this respect confirms earlier permeability studies in a wide range

of lipids (5) and extends a recent spin label study with halothane in cholesterol containing bilayers (8). The previous results (7) might be in error through failure to adequately control the volatile anesthetics studied or to the inherently large errors associated with electron spin resonance work. The latter point is underscored by the comparison of methods presented in Fig. 3.

The membrane concentrations corresponding to anesthesia may be calculated. Using the buffer concentrations summarized in a previous paper for general anesthesia in newts and goldfish (5), together with a recent figure for pentobarbital in tadpoles (37), the corresponding mole fractions in our bilayer are 0.044, 0.041 and 0.019 for halothane, butanol and pentobarbital, respectively. Values given for nerve block by Seeman (12) are higher than for general anesthesia and yield corresponding mole fractions of 0.37, 0.14 and 0.17. Using the valinomycin data, the changes of permeability at general anesthetic concentrations are respectively 1.5, 1.5 and 1.2 times, and at nerve block 28, 3.7 and 5.0 times, control values. Thus quite clearly measurable changes in permeability are observed in each case, while those related to nerve blocking concentrations are substantial. There remains little doubt that permeability, as well as order parameter (8) changes in bilayers are detectable at physiological concentrations, although in the case of general anesthesia these changes are small.

Table 1 shows that at equal membrane concentrations all the anesthetics produced very similar effects on each mode of ion transport including that with no ionophore present. The underlying perturbation, thus, probably occurs within the lipids and does not represent an ionophore-anesthetic interaction. This perturbation is not markedly dependent on the anesthetics' structure but is related to their lipid solubility. The coupling between this apparently uniform perturbation and all the ion fluxes is very similar, although somewhat less effective with gramicidin A than valinomycin.

The question of coupling between lipid perturbation and membrane function is now central to development of the fluidized (or expanded) lipid hypothesis of anesthetic action. Our fluorescence data, when plotted against membrane concentration, yields a straight line. Taking a mole fraction of 0.02 to 0.04 as the anesthetic concentration the change in microviscosity is 2½–5%. Similar considerations at nerve blocking concentrations yield changes of 16–42%. For halothane a value of the partial molar volume in egg phosphatidylcholine is available (Bennett & Miller, unpublished data) and the calculated expansion is 1% for anesthesia and 8% for nerve block in these bilayers. Our spin-label study with butanol yields an order parameter change of 2% at general anesthetic concentrations and 7% at nerve block (Fig. 3). In corroboration of the order of magnitude of these changes an order parameter change of about 1% at an anesthetic dose of halothane was reported in a careful study of egg phosphatidylcholine: cholesterol liposomes (8). Thus at general anesthetic concentrations a 2–5% change in structural parameters produces a 20–50% change in ion permeability. The functional change is about an order of magnitude larger than the associated structural perturbation in the lipids.

Are such changes as these sufficient to account for anesthesia? Such a question is hard to answer on the present information. First, the ionophores studied here are very simple; more complex ones, such as alamethicin with its potential dependent "gate," might couple to the lipid perturbation much more strongly. Second, the boundary lipid close to physiological ionophores, such as rhodopsin (38) or Ca^{2+}-Mg^{2+} ATPase (11), might be more or less perturbed by anesthetics. On the other hand, the anesthetic induced change in time constants of the decay phase of miniature end-plate currents at the neuromuscular junction is only about a factor of two at blocking doses (39). Thus, the observed magnitude of the changes in simple bilayer systems does not currently provide any evidence for rejecting the lipid perturbation model. Unequivocal support for the model can, of course, only come from work on physiological systems, but studies on more complex model ionophores could provide a

further test.

We now consider the nature of the lipid perturbation that influences the permeability by considering spectroscopic evidence on anesthetic-lipid interactions. These three anesthetics were chosen to reveal different aspects of the interaction. Halothane is relatively nonpolar, is evenly distributed through the bilayer (40), decreases the order parameter of spin probes at different depths in the bilayer (6, 7) and decreases the microviscosity of the hydrocarbon interior. Butanol is similar but on average probably distributes preferentially into the bilayer's interface because of its hydrogen bonding capacity (34). Pentobarbital is probably also amphiphatic, and most significantly, it is the only anesthetic studied here which increases the order parameter reported by spin-labeled lipid probes (10, 23, 41). This ordering effect is stronger at the fifth than at the eighth acyl carbon. Spin-labels deeper in the bilayer yield little information because of the high fluidity, but DPH fluorescence depolarization reveals a decrease in microviscosity. Thus the observed changes in permeability correlate best with the changes in microviscosity derived from the fluorescence depolarization of DPH for these anesthetics. This strongly suggests that the underlying lipid perturbation resides in the interior of the bilayer in each case. The lack of correlation between ion permeability and order parameter in lipid vesicles has also been noted for a number of steroids (42, 43).

The correlation of the anesthetic effects with hydrocarbon fluidity recalls the established effects of increasing acyl unsaturation and decreasing cholesterol content in increasing valinomycin mediated cation fluxes (27, 28, 44). It has also been observed that the opposite effects of anesthetics and pressure on valinomycin mediated cation permeability correlated with expected changes in bilayer density (5). Further work may show the density and microviscosity descriptions to be equivalent to some degree. The advantage of the density description is that it is a thermodynamic quantity whereas the spectral parameter is more difficult to interpret precisely. The correlation of these structural parameters with anesthetic action on ion fluxes does not imply that they are directly related to the mechanism of the anesthetic effect. Other properties which are co-variant, such as dielectric constant, may well be more relevant. More detailed studies will be required to answer such questions.

It has recently been shown that anesthetics decrease the overall conductance observed in black lipid membranes in the presence of a gramicidin derivative by affecting the number of channels conducting, with unchanged unit conductance, at a given time (18). These effects have been related to changes in bilayer thickness and tension. We found the effects of anesthetics on the effects of gramicidin mediated cation flux in liposomes to be in the opposite direction. If the rate limiting step in our work is the transfer of gramicidin between vesicles, these observations are not contradictory. However, if channel duration is rate limiting, the discrepancy between the liposome and planar bilayer work might be resolved by the recent observation that the thickness of black lipid membranes is increased by anesthetics while that of liposomes is changed little (45) or, as many authors have predicted, decreases slightly (46, 47). Studies in "solvent-free" planar bilayers may yet resolve these uncertainties and provide more detailed insights of the mechanisms involved (48, 49).

ACKNOWLEDGMENTS

We thank Dr. J. Gergely, A. K. Solomon and A. Kleinfeld for use of spectroscopic equipment.

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MOLECULAR PHARMACOLOGY, 18, 84-90

The Perturbation of Lipid Bilayers by General Anesthetics: A Quantitative Test of the Disordered Lipid Hypothesis

KAM-YEE Y. PANG, LEON M. BRASWELL, LARRY CHANG, TOBY J. SOMMER AND KEITH W. MILLER

Departments of Anaesthesia and Pharmacology, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts 02114

Received June 22, 1979; Accepted January 21, 1980

SUMMARY

PANG, K.-Y. Y., L. M. BRASWELL, L. CHANG, T. SOMMER AND K. W. MILLER. The perturbation of lipid bilayers by general anesthetics: A quantitative test of the disordered lipid hypothesis. *Mol. Pharmacol.* 18: 84-90 (1980).

The ability of a wide range of general anesthetics to perturb the order reported from spin-labeled phospholipid:cholesterol (2:1) bilayers has been examined. The change in order induced by increasing concentrations of the following were examined: ethanol, butanol, trichloroethanol, α- and β-chloralose, urethane, pentobarbital, thiopental, ketamine, and phenytoin. All except the latter and β-chloralose caused marked decreases in order. The bilayer/buffer partition coefficients of phenobarbital, phenytoin, and urethane were measured. The change-in-order parameter as a function of total anesthetic concentration varied widely but when the agents were compared at constant concentration in the bilayer all the anesthetics examined gave very similar values. Phenobarbital was somewhat more effective at disordering than the other barbiturates. Phenytoin's weak disordering ability was probably due to solubility limitations rather than an inability to disorder. When the general anesthetic and nerve-blocking potency of these agents were compared to their membrane disordering ability, fair correlations were obtained, but the barbiturates tended to deviate and this deserves further attention. Furthermore the change-in-order parameter at general anesthetic concentrations is only 0.6% which is small compared to the variation to be expected in the physiological temperature range. Thus although the disordered lipid hypothesis is fairly successful at correlating the anesthetic potency data over a dose range of four orders of magnitude, some problems remain. How far these can be overcome by developing more realistic models within the framework of the hypothesis remains to be seen.

INTRODUCTION

Circumstantial support for the lipid theories of general anesthetic action has remained strong since the turn of the century (1). In the last decade advances in the understanding of membranes have been reflected in a renewed interest in these theories. The advent of lipid bilayers in particular has enabled the lipid–anesthetic interaction to be examined in greater detail. Gaseous, volatile, alcohol, steroid, amine, and barbiturate general anesthetics have all been shown to disorder lipid bilayers (2–7), although the last two classes of anesthetics only do so if certain proportions of cholesterol are included in the phospholipid bilayer (6). These changes are opposed by pressure (5, 8, 9), as is anesthesia with these agents (10, 11), and do not occur with the lipid-soluble nonanesthetic long-chain alcohols (4). The measured order parameter changes at anesthetic concentrations are close to the limit of detection of the method (5) but can be shown to be real (12).

In spite of this qualitative success few attempts have been made to quantitatively test the disordered lipid hypothesis of anesthetic action. In the case of gaseous and volatile agents the gas phase may be used as a reference state and some quantitative comparisons with anesthetic partial pressure have been attempted (9). In other cases where quantitative correlations have been attempted (7) considerable doubt remains because in the absence of lipid/buffer partition coefficients the distribution of anesthetic between the lipid dispersion and that free in solution is undefined. With this in mind we have measured the ability of nine general anesthetics, ranging from alcohols to amines, and one anticonvulsant

K. W. M. is a Research Career Development Awardee of the National Institute of General Medical Sciences (GM 00199). This work was supported in part by a grant from NIGMS to the Harvard Anaesthesia Center (GM 15904) and in part by joint funding from the Office of Naval Research and the Naval Medical Research and Development Command through Office of Naval Research Contract N00014-75-6-0727.

0026-895X/80/040084-07$02.00/0

to disorder egg phosphatidylcholine:cholesterol (2:1) bilayers. This level of cholesterol was chosen because we, and others, have previously found bilayers with less cholesterol are not always disordered by anesthetics (6, 13, 14). For some of these compounds the partition coefficients are known, but we have determined them for some of the others. For these compounds we are thus in a position to approach two quantitative questions. First, since we can calculate the free aqueous concentration of anesthetic, we are able to compare the aqueous concentrations required to cause general anesthesia or to block axonal conduction. Good correlations are found for most of this structurally diverse group of agents. Second, since the actual amount of anesthetic in the lipid is known, the ability of all the agents to change the order parameter at a given membrane concentration, a quantity we defined as disordering efficacy (6), can be examined. In this lipid bilayer the anesthetics all have roughly equal efficacies, and the apparently lower efficacy of the nonanesthetic phenytoin probably arises from solubility limitations.

MATERIALS AND METHODS

The effect of various general anesthetics and the anticonvulsant on membrane structures was generally monitored from the electron spin resonance (ESR) spectra of 1-acyl-2[8-(4,4-dimethyloxazolidine-*N*-oxyl)]palmitoylphosphatidylcholine [PC[1] (7, 6)], which was synthesized in this laboratory by Dr. M. Pringle according to the method of Hubbell and McConnell (15). Egg yolk phosphatidylcholine and phosphatidic acid were from Lipid Products, Surrey, United Kingdom, and used without further purification. Cholesterol (Sigma Chemical Co., St. Louis, Mo.) was recrystallized in methanol. Phenobarbital, thiopental, and phenytoin were from Sigma. Ketamine hydrochloride was a gift from Dr. R. M. Wheelock, Parke-Davis and Company, Detroit, Michigan. Octanol was purchased from Applied Science, State College, Pennsylvania. Trichloroethanol, chloralose and urethane were Aldrich products (Milwaukee, Wisc.).

The method for preparing vortexed phosphatidylcholine:cholesterol liposomes for spin-label studies has been reported previously (13). Drugs were codeposited with lipid before addition of buffer solution (0.01 M Tris–HCl, pH 7.0, in 0.1 M KCl). For the highly lipophilic agents, such as phenobarbital and phenytoin, their low solubility in the aqueous phase made it necessary to pay special attention to ensure that all drug was incorporated into the dispersion. In some cases radiolabeled tracers were employed for this purpose. The highly water-soluble agents, for example, urethane, were dissolved in the buffer as well as added to the lipid film to form liposomes of the desired total lipid to drug ratio. Volatile agents were only added in the buffer. Four percent of the phospholipids were always phosphatidic acid. The order parameter was calculated as previously (13) with polarity and $T_\perp$ corrections from the ESR spectra determined at 25°C on a Varian E-109 spectrometer. The disordering efficacy of an anesthetic is defined as the negative of the change-in-order parameter per unit concentration of anesthetic in the membrane (units of mM^{-1}).

[1] Abbreviation used: PC, phosphatidylcholine.

Partition coefficients of phenobarbital and phenytoin (^{14}C labeled, New England Nuclear) were measured using the ultrafiltration method of Miller and Yu (16). Incubations were carried out at 25°C with 2 mg/ml of lipid and 0.07 mM total concentration of drug. The pH was adjusted to give 90–95% association of these weak acids. Urethane's partition coefficient is too small for the ultrafiltration technique. The centrifugation method of Katz and Diamond (17), including corrections for nonsolvent water in the pellet, was used. Samples containing lipid, [^{14}C]urethane, and [^{3}H]sucrose (New England Nuclear) were incubated overnight at 4°C and then for 6 h at 25°C and pH 7.0 before centrifugation. All partition coefficients are expressed as (moles of anesthetic per milliliter of lipid/moles of anesthetic per milliliter of buffer). The ED_{50} for loss of righting reflex in tadpoles was determined as previously described (18).

RESULTS

The results of the spectroscopic measurements are presented in Figs. 1a and b, as plots of the change-in-order parameter of the spin-label PC (7, 6) as a function of the ratio of total moles of drug to phospholipid. The control order parameter had an average value of 0.62 but varied ±4% from preparation to preparation. In spite of this variation, which may arise from lipid oxidation, the value of ΔS observed in the presence of anesthetic remained relatively constant (4). In most cases two or more independent experiments were performed on each anesthetic with several different concentrations. Error bars are omitted from the diagram for clarity, but reproducibility in the ΔS values from day to day was generally within ±0.01.

Phenobarbital produced the strongest disordering effect at a given total concentration, although the maximum effect attained was less than with many other agents. Thiopental was similar but its effect seemed to saturate, probably due to limited solubility (the point at highest concentration was not included in the regression in Fig. 1a). Ketamine and trichoroethanol had similar slopes, but the alcohol exerted the larger maximum effect (Fig. 1b). α-Chloralose and butanol also had similar but smaller slopes, and the alcohol again produced the larger maximum effect. β-Chloralose was examined, but this nonanesthetic had such limited solubility that no significant changes were recorded. Urethane and ethanol had the lowest slopes of all the anesthetics. Although the alcohol once more exerted the highest maximum effect, this was only attained at concentrations so high that the data do not even appear on the extended scale of Fig. 1b. They are therefore given in the legend. Finally for comparison the data for the anticonvulsant, phenytoin, are shown. It had a slope between those of butanol and urethane, but the largest decrease in order parameter it produced was smaller than that of any other agent. Concentrations higher than those examined could not be dissolved.

Thus when considered on a total concentration basis the anesthetics fail to produce a consistent decrease in lipid order. This is probably an artifact of the method of comparison, because the slopes of the regressions in Fig. 1 tend to increase with increasing lipophilicity. Indeed

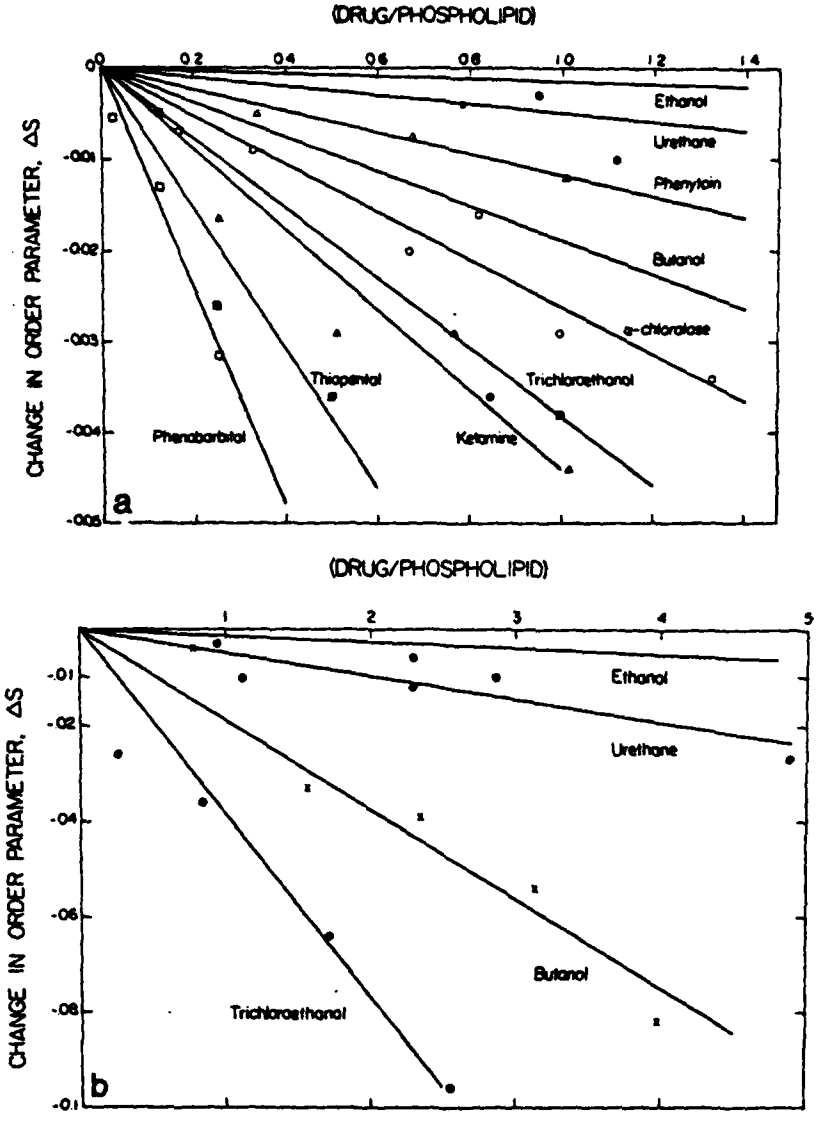

FIG. 1. *Plots of change-in-order parameter of the spin-label PC*

(a) The change-in-order parameter reported by PC (7, 6) as a function of the mole ratio of drug to phospholipid in vortexed lipid dispersions (64.3 mol% egg phosphatidylcholine; 2.7 mol% egg phosphaditic acid; 33 mol% cholesterol). Phospholipid concentration was usually 40 mM. The lines are least-squares fits through the origin, but not all the data necessarily are on the graph (see Fig. 1b). Key (agent, symbol, slope ± SD (no. of points)): ethanol, +, −0.0013 ± 0.00022(3); butanol, ×, −0.019 ± 0.0013(5); trichloroethanol, ●, −0.038 ± 0.0030(4); α-chloralose, ○, −0.026 ± 0.0016(6); phenobarbital, □, −0.120 ± 0.0085(3); phenytoin, △, −0.012 ± 0.0012(4); thiopental, ■, −0.076 ± 0.011(3), highest point omitted, see text; ketamine, ▲, −0.044 ± 0.0040(4); and urethane, ●, −0.0048 ± 0.00059(6). For ethanol and butanol 8-doxylstearic acid was used instead of PC (7, 6). (b) As in (a) but the scale is extended to include all data omitted in that figure except those for ethanol which were: 15.6, −0.0017; 31.3, −0.057; and 47, −0.053 (drug/phosphate, ΔS).

ethanol has a lipid/buffer partition coefficient of less than one and thus most of the ethanol will be in the aqueous phase because only a few percent by volume of our suspensions are lipid. Even with the more lipophilic agents examined about a fifth of the anesthetic remains in the aqueous phase. Thus although Fig. 1 is a convenient way of presenting the data, knowledge of the partition coefficients would enable a more meaningful analysis to be made.

The partition coefficients of phenytoin and phenobarbital in 2:1 egg phosphatidylcholine:cholesterol were found to be 330 and 83, respectively (for the completely associated acids). In the absence of cholesterol these values rose to 657 and 125, respectively, an effect similar to that noted previously with pentobarbital and thiopental. The overall accuracy of these figures is about 5% (16). Using the centrifugation assay the partition coefficient of urethane in the mixed lipids had a mean value of 1.09_5 with a variation of 1% between the two runs.

These partition coefficients enable some of the data in Fig. 1 to be recalculated to allow for the partitioning of anesthetic between lipid and buffer. A partition coeffi-

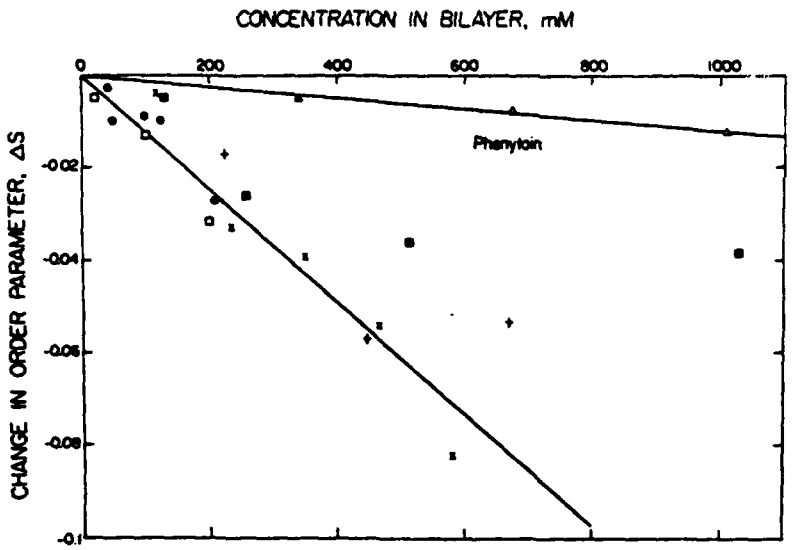

FIG. 2. *The change-in-order parameter for five anesthetics and phenytoin presented as a function of the concentration of the anesthetic in the bilayer*

Key as in Fig. 1. Sources of the partition coefficient data used in the calculation of bilayer concentration are given under Results and in Table 1. The slope of the line is $-1.2 \pm 0.13 \times 10^{-4}$ mM^{-1} for the five anesthetics and $-0.12 \pm 0.01 \times 10^{-4}$ mM^{-1} for phenytoin. The two highest concentrations of thiopental, ■, were supersaturated and therefore omitted from the regression (see Results).

cient for thiopental is also available (Korten and Miller, unpublished data) and we have used data (17) for ethanol and butanol in dimyristoylphosphatidylcholine above its phase transition to extend our calculations approximately to these agents. The results of these calculations are given in Fig. 2. A number of points must be considered in assessing these data. First, the data for the alcohols are least reliable. Not only are the partition coefficients in a different lipid, but the spectroscopic data were obtained with 8-doxylstearic acid instead of PC (7, 6). Therefore the very good fit of the alcohol data with the other anesthetics should not be emphasized. We have included the data to underscore the importance of considering membrane concentration for these agents of low partition coefficient. Second, the calculation shows that the final aqueous concentration exceeds the experimental solubility limit which we determined in our buffer system in some cases. The two highest concentrations of thiopental were omitted from the regression for this reason, they actually exceed the saturation limit by two and a half and five times. It is probable that some of the biphasic effects reported previously for thiopental (19) resulted from exceeding the solubility limit. The nonanesthetic, phenytoin, exhibits a different slope in Fig. 2. However, at all concentrations saturation was again exceeded. Since the spread of the data is small compared to our errors, the apparent trend with concentration could be fortuitous and actually represent a saturated solution in each case with the excess phenytoin undissolved. The concentration in lipid at saturation is 25–30 mM, which would bring the data close to the regression line for the other agents. On the other hand phenytoin might be incorporated into the lipid above the saturation limit if some form of phase separation were to occur. The observed effects were too close to the resolution of the method to make further investigation worthwhile.

ED_{50}'s for tadpole loss of righting reflex were found as follows (±SE); 170 ± 22 μM for ketamine; 115 ± 11 mM for ethanol; 12 ± 1.4 mM for butanol; and 1.8 ± 0.23 mM for α-chloralose. Phenytoin and β-chloralose failed to cause anesthesia even at saturation.

DISCUSSION

Disordering efficacy. Comparison of Figs. 1 and 2 demonstrates for the first time that agents with a 400-fold range of lipid to buffer partition coefficients are equally effective at disordering phospholipid:cholesterol (2:1) bilayers when compared on a membrane concentration basis. The only exception is phenytoin, but this is more apparent than real since all the data were obtained above the solubility limit. It must be emphasized that this conclusion applies only to the perturbation of order at the eighth acyl carbon that we have measured. It seems unlikely, for example, that 1.5 M ethanol (our highest free aqueous concentration) would fail to modify dipolar interactions in the lipid–aqueous interface.

The conclusion for the five anesthetic agents in the present work (Fig. 2) can be extended to include six more agents (Table 1) as follows. Order parameter data for xenon and halothane using the same lipid bilayer and spin label and using the vapor phase as standard state have been given by Trudell and colleagues (9, 12). The data for xenon were determined at elevated pressure, but this can be corrected (9). Using a partition coefficient for halothane (12) and xenon (Smith and Miller, unpublished data) the disordering efficacies in Table 1 were calculated by plotting ΔS versus membrane concentration. Similarly we were able to add data for pentobarbital (13, 16).

For the remaining three anesthetics we examined, no partition coefficient data are available. However, we noticed for a number of agents for which we have partition coefficients in egg phosphatidylcholine:cholesterol (2:1)

TABLE 1

Disordering Efficacy of Eleven General Anesthetics

The disordering efficacy was calculated as described in the Discussion. It is defined as the negative of the change-in-order parameter per unit concentration of anesthetic in the membrane. For agents 1–6 the bilayer/buffer partition coefficients are known in phosphatidylcholine: cholesterol (2:1). For the subsequent agents the partition coefficients were approximated by using for agents 7 and 8 partition coefficients in dimyristoylphosphatidylcholine (17) and for the remaining agents octanol/buffer partition coefficients (see Discussion). Order parameter data are from this work, except those for pentobarbital (13), xenon (9), and halothane (12).

Number	Agent	Disordering efficacy, ΔS (10^{-4} mM)	Partition Coefficient (pH 7.0) (Source)
1	Thiopental	0.85	172 (Unpublished)
2	Phenobarbital	1.53	51 (This work)
3	Pentobarbital	1.02	56 (16)
4	Urethane	1.15	1.1 (This work)
5	Xenon	0.91	14 (Unpublished)
6	Halothane	1.75	50 (12)
Mean ± SD (1–6)		1.2 ± 0.36	
7	Ethanol	0.93	0.44 (17)
8	Butanol	1.27	3.2 (17)
Mean ± SD (1–8)		1.2 ± 0.32	
9	α-chloralose	0.39	40 (20)
10	Ketamine	0.40	400 (calculated (21))
11	Trichloroethanol	1.66	7.1 (calculated (21))
Mean ± SD (1–11)		1.1 ± 0.46	

that the ratio of the octanol/buffer to the lipid/buffer partition coefficient varies from 0.3 to 2.4 with values for 80% of the agents between 0.7 and 1.8. We have therefore used the octanol partition coefficients (20, 21) of these three anesthetics to estimate their membrane concentration and hence their disordering efficacy.

Thus for six anesthetics, including an inert gas, a carbamate, and barbiturates, we have summarized in Table 1 the measured value of the disordering efficacy. It has a mean value of 1.2×10^{-4} mM^{-1} with a standard deviation of only 27%. The total range of efficacies is 2-fold between halothane and thiopental. Within the present work it is 1.8-fold between phenobarbital and thiopental. Such a range is probably higher than the combined errors of measuring order parameters (individual regressions of ΔS versus concentration generally had a standard deviation of 10%), phospholipid concentration (2%) and partition coefficients (5%), together with errors due to failure to incorporate anesthetic or lipid into the suspension. Thiopental and pentobarbital, differing only in the substitution of a sulfur for an oxygen atom at the second carbon in the pyrimidine ring, have efficacies which differ by no more than the expected errors. On the other hand phenobarbital with its bulkier substituent on the fifth carbon in the ring has a significantly higher disordering efficacy. Urethane (ethyl carbamate) has an efficacy similar to that of the former two barbiturates. The precision with which the xenon and halothane data can be expected to compare to our data is unclear, but it is surprising that halothane is a more effective perturber than either xenon or most of the agents we examined. Whether this is caused by its high polarity, the weakness of fluorocarbon–hydrocarbon interactions or other causes is uncertain.

The disordering efficacies of the two alcohols hardly differ from each other or from the preceding six agents in spite of the approximation made in the partition coefficient used, but the agreement must be regarded as partly fortuitous. The three remaining anesthetics in Table 1 all have disordering efficacies which probably do not differ from those of the other agents when the crude method for estimating their bilayer concentration is taken into account.

Thus, the overall conclusion to be drawn from Table 1 is that the disordering efficacy of a diverse range of anesthetics in phospholipid:cholesterol bilayers varies little. Two additional points are of interest with respect to this conclusion.

First, the conclusion is not independent of membrane composition. For example, in the absence of cholesterol pentobarbital has a negative disordering efficacy (it orders) while halothane still has a positive disordering efficacy (6). We have discussed possible reasons for the change in sign of the efficacy that addition of cholesterol produces in a previous paper (13). It appears here that 33 mol% cholesterol is sufficient to completely mask any such effects exhibited by these agents at lower cholesterol contents. For example, pentobarbital changes from ordering to disordering at 14 mol% cholesterol (13).

Second, do the lipid-soluble nonanesthetics and partial anesthetics have low disordering efficacy, as has been suggested (4), or are they simply solubility limited? Our data show that for the nonanesthetic, phenytoin, solubility limits the absolute decrease in order. The maximum effect is too small to be reliably measured, and therefore we are unable to comment on its disordering efficacy which may be less than, comparable to, but not much larger than that of the other agents. A similar situation holds for hexadecanol (Pringle and Miller, unpublished data). Thus solubility limits are a sufficient explanation for the lack of potency for these two agents. In other cases this may not be so. Thus for the partial anesthetic, tetrahydrocannabinol, we found that order increased for total drug to phospholipid ratios of 0.06 to 0.20 with a maximum increase in order parameter of +0.02. This is the only agent for which we have recorded ordering at such high cholesterol content in egg lecithin, and the only one for which the efficacy appears to differ from the other agents.

The disordered lipid hypothesis of anesthetic action. We now turn to using the information summarized in Table 1 to test the hypothesis that anesthetic action correlates with changes in lipid order reported from the eighth acyl carbon of phosphatidylcholine incorporated in a bilayer with 33 mol% cholesterol. This means that the product of disordering efficacy and the membrane concentration which causes anesthesia is constant for all anesthetics. To the extent that disordering efficacy is constant we are simply testing the relation between

bilayer solubility and anesthetic action. This is analogous to the problem of distinguishing the lipid solubility and expansion theories, where, as here, it is necessary to invoke the pressure reversal of anesthesia to justify the inadequacy of the solubility theory (22). It is unfortunate that none of the full anesthetics examined has such an anomalous disordering efficacy that the lipid solubility and disorder hypotheses might be distinguished on this basis alone.

To perform this test conveniently we have calculated from our data the aqueous concentration of each anesthetic which at equilibrium causes an arbitrary change-in-order parameter of −0.01. In Fig. 3 this quantity is seen to correlate well both with the concentration required to anesthetize tadpoles and with a set of data for block of a compound action potential in nerve. Sources of data are given in the legend. Each set of data was fitted by the best least-squares line both freely (dashed line) and also constrained to a slope of 1.0 (solid line) as required by the hypothesis.

The data for 10 anesthetics freely fitted to the tadpole anesthesia results yielded a slope of 0.8 ± 0.11. There are two major deviations from the hypothesis line; thiopental is eight times more, and phenobarbital five times less, potent than predicted. If these two agents are omitted the mean change-in-order parameter for general anesthesia is −0.0035 ± 0.0014, which is similar to a previous estimate for halothane alone (12). Thus for 8 out of 10 anesthetics a satisfactory fit is achieved covering four orders of magnitude of potency; these agents include an inert gas, two alcohols, an amine, a barbiturate, and a volatile anesthetic, as well as a carbamate and α-chloralose. Although this correlation could arise by chance, the fact that no such correlation would be found if cholesterol were omitted from the bilayer tends to argue against this. Similar correlations have been presented for a homologous series of alcohols (7). The barbiturates as a class, however, deviate markedly from our correlation and deserve further attention.

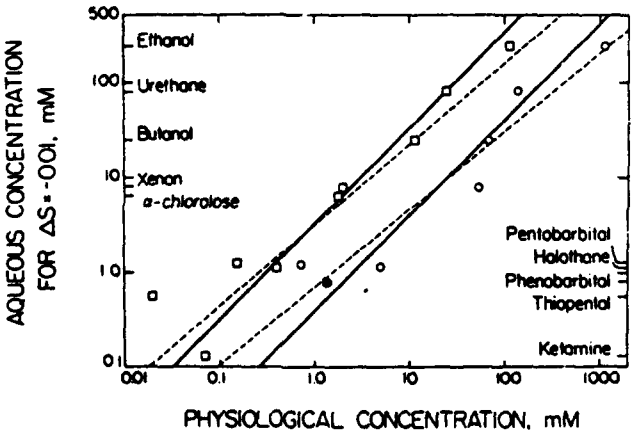

FIG. 3. *Correlations between physiological potency and disordering effect*

The aqueous concentration corresponding to a change-in-order parameter of −0.01 was calculated from the data in Table 1. The nerve block data, ○, were for sciatic nerves from Refs. (23, 24). Tadpole anesthesia data, □, from (23, 25) and this work. All concentrations are for the uncharged form of the drug. The dashed lines are least-squares fits and the solid lines were fitted similarly but with the slope constrained to one.

The set of data for block of the compound action potential in sciatic nerve fitted the order parameter data with a slope of 0.8 ± 0.11. The mean change-in-order parameter at nerve block is −0.03 ± 0.023. The largest deviation from the hypothesis line (slope = 1.0) is fourfold for pentobarbital. There is one other deviation, however, which is not shown. Phenytoin blocks at close to saturation (24) where we expect the order parameter change to be less than −0.01. Thus overall the data fit the model for nerve block fairly well but the use of the compound action potential which represents the sum of several processes (26), and does not take into account the frequency dependence of block noted in some instances (27), is less than ideal. However, no other uniform set of data is available for such a wide range of agents.

It would be premature to reject the model on the basis of the deviations noted above. Thus the ability of barbiturates, but not the volatile agents, to perturb bilayers is sensitive to both their cholesterol and negatively charged lipid content (6, 13). Probably the incorporation of protein into the bilayer would further modulate their disordering efficacy. The model might thus be fine-tuned by adjustments of the membrane composition.

Some other indications that the model would benefit from such fine tuning are that the partial anesthetic tetrahydrocannibinol orders this bilayer, has little influence on a bilayer with 50 mol% cholesterol (Pang and Miller, unpublished data), but does disorder a bilayer with the saturated phospholipid, dipalmitoylphatidylcholine, and cholesterol (1:1) (28). Similarly nitrogen does not disorder the present model (9) but does disorder red cells under some circumstances (29), an indication that membrane protein may also modulate disordering efficacy.

One other problem of the model deserves mention. The order parameter change equivalent to anesthesia is only −0.0035, or a relative change of 0.6%, which is similar in magnitude to the percentage volume change found for the critical volume hypothesis (30), as might be expected for two interrelated variables (8). Our control experiments show that a similar change-in-order parameter can be produced by raising the temperature 0.32°C. This clearly is a problem for the disordered lipid hypothesis, as has been recently emphasized (31). However changing lipid order by introducing an anesthetic into the lipid bilayer is qualitatively different from doing so by introducing thermal energy. The latter perturbant will be sensed by all parts of the system, and the change in order might well be compensated for elsewhere in the perturbation–response chain. However, this remains to be demonstrated. It is interesting to note that the critical volume hypothesis does not suffer from this disadvantage. Its critical volume is equivalent to an increase in temperature of over 10°C (30). It is thus possible that membrane volume changes are a more appropriate model for anesthesia than lipid order.

Thus, we conclude that the disordered lipid hypothesis of anesthetic action correlates the activity, or lack of it (phenytoin and β-chloralose), of a diverse set of agents

whose potencies range over four orders of magnitude. The hypothesis has the least success with the barbiturates. Although there are grounds to believe that refinement of the model might remove the latter anomalies, this remains to be proven. Since the disordering efficacies of all the agents examined were similar our data fail to distinguish between a disordered lipid and a simple lipid solubility model, although other criteria, such as pressure reversal of anesthesia, could do so. The importance of directly determining lipid solubility is emphasized by our findings for phenytoin. Had we found the latter to be present in bilayers that were little disordered the disordered lipid hypothesis would have been supported. As it was we found solubility to be a sufficient criterion for lack of anesthetic potency. Thus the supposed support for the disordered lipid hypothesis based on other nonanesthetic lipophilic agents which did not disorder bilayers (4) may need to be reassessed. This will require the measurement of appropriate partition coefficients. These arguments do not detract from the overall success of lipid-based unitary theories of anesthesia in general. They do leave open the question whether disorder in lipid acyl chains is an appropriate operator, particularly in view of its temperature dependence. Any other variable which is proportional to lipid solubility can satisfy pressure reversal by hydrostatic pressure and helium, and is less sensitive to temperature could also be appropriate. We have reviewed a number of possibilities in a previous paper (32).

ACKNOWLEDGMENT

We wish to thank Dr. J. Gergeley, Boston Biomedical Research Institute, for use of election spin resonance equipment.

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Send reprint requests to: Dr. Keith W. Miller, Department of Anaesthesia, Massachusetts General Hospital, Boston, Mass. 02114.

Molecular Mechanisms of Anesthesia (Progress in Anesthesiology, Vol. 2), edited by B. Raymond Fink. Raven Press, New York © 1980.

Action of Anesthetics and High Pressure on Cholinergic Membranes

J. Francois Sauter, Leon M. Braswell, and Keith W. Miller

Departments of Anesthesia and Pharmacology, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts 02114

INTRODUCTION

Studies of the mechanism of anesthesia, per se, suffer from the major problem that the site of anesthesia remains unknown. Thus an indirect approach has often been adopted. The predictions of proposed models of anesthetic action are compared with the physiological data for some behavioral end-point, such as loss of righting reflex or failure to respond to a painful stimulus. This black-box approach has been remarkably successful in distinguishing various models from one another (14). This is partly due to the high precision with which anesthetic potency can be measured, which is the result of steep dose-response curves and the ability, at equilibrium, to measure the dose in the central nervous system (CNS) as the partial pressure of anesthetic applied. Such studies have thus been limited to volatile agents, since the use of intravenous agents is fraught with pharmacokinetic artifacts. Two important limitations to this method must be recognized. First, the predictions of a model can only be shown either to be unable to fit the data or to be consistent with it. Thus models may be rejected but never proved. Secondly, models must be simple enough to allow unambiguous predictions to be made objectively.

Currently the most successful of such models is the critical volume hypothesis, which states that anesthetics act by dissolving in and expanding some hydrophobic phase or site (14). When this expansion exceeds a certain critical value, anesthesia results. Pressure reverses anesthesia by opposing this expansion. Available solubility data can be used to model the hydrophobic site and the choice of solvent can be made objectively; the only remaining adjustable parameter is the compressibility, but even this is found to fall within the rather narrow range of physically realistic values. Using this model, the solvent power, or solubility parameter, and the compressibility of the site of action may be characterized (17). Two questions remain unanswered: Does the success of this model arise by chance? What is the nature of the hydrophobic phase that is so well modeled by organic solvents?

On the latter point, much evidence is consistent with the hydrophobic site being the lipid bilayer of some neural membrane. The solubility of gaseous and volatile anesthetics in lipid bilayers closely parallels that in organic solvents (13). The ion permeability of lipid vesicles is increased by anesthetics and decreased by pressure in a manner quantitatively in accord with the critical volume hy-

pothesis (8). Detailed spectroscopic studies on lipid order have been carried out on the premise that lipid expansion and disordering are equivalent (7). These studies show that all anesthetics disorder bilayers, partial anesthetics partially disorder bilayers, and lipid-soluble nonanesthetics do not disorder bilayers (11,22). Pressure orders bilayers, opposing the effect of anesthetics (22). In order for the model to be successful it is necessary to include cholesterol in the bilayers. For example, pentobarbital orders bilayers lacking cholesterol (16). At least 30 mole % of cholesterol is required to ensure that most anesthetics disorder the bilayer. This value is interesting, since nerve membranes have similar cholesterol contents (18).

Thus, these black-box models have been very successful. Although quite plausible alternative models exist in principle, they are usually incapable of making predictions that are quantitatively testable in this way. For example, in a recent publication on the degenerate perturbation hypothesis, which proposes that anesthetics may bind directly to many sites of different structural specificity on an excitable protein, all the data presented were on lipid-anesthetic interactions (19).

In order to avoid the limitations of the black-box models it is necessary to examine specific biological systems. It is important to acknowledge that in so doing one is no longer studying general anesthesia. General anesthetics are nonspecific agents of low affinity and it is quite plausible that they may produce effects at different locations by totally unrelated mechanisms. Indeed the data to be presented here suggest that at a single synapse more than one mechanism may be at work.

When such specific systems are studied it is often possible to test models in detail. Thus Adams has produced evidence consistent with the idea that barbiturates selectively bind to a site on open ionophores in the frog end-plate (1), whereas others have implicated lipid fluidity in the action of volatile agents at the neuromuscular junction (5). Biochemical studies have shown that barbiturates displace the saturable binding of picrotoxinin to rat brain membranes (20).

One criterion for evaluating the relationship between such observations and the actions of the same agents in producing general anesthesia is pressure reversal of the anesthetic effect. A second rationale in our study was that it had never been demonstrated that pressure and anesthetics could oppose each other's effects at a single synapse; yet this is a fundamental assumption of many of the black-box models. We wished, then, to examine the actions of anesthetics on a single synapse and to elucidate the mechanisms of such actions and the ability of pressure to modulate them. We chose to do so in acetylcholine (ACh) receptor-rich membranes isolated from the electroplaque of *Torpedo californica.* This rich source of material enables many types of study to be undertaken, and the high specific activity allows structural perturbations to be measured also. Previous chapters in this volume have provided examples of such studies.

STUDIES ON THE EQUILIBRIUM BINDING OF ACETYLCHOLINE TO ITS RECEPTOR

ACh receptor-rich membranes were isolated from freshly killed *Torpedo* by centrifugation (3). The material obtained had a specific activity of 1 to 3 μmoles of ACh sites and 0.5 to 1.0 mmoles of phosphate per gram of protein. The major lipids are phosphatidylcholine, phosphatidylethanolamine, and cholesterol, the ratio of phospholipid to cholesterol being close to 1:1 (18).

The effects of anesthetics were first examined on the equilibrium binding of [^{3}H]ACh, using a centrifugation assay (4). AChR stock solution, 1.2 ml (3μM, in sucrose) is diluted to

13.5 ml with *Torpedo* Ringer (250 mM NaCl, 5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7) and mixed with 1.5 ml of 10^{-3}M DFP. One-milliliter aliquots are added to 12 polycarbonate centrifuge tubes and α-bungarotoxin added where appropriate. After 30 min (independent controls show these conditions block all acetylcholine esterase (AChE) activity) 6.8 ml of Ringer containing anesthetics as appropriate are added to each tube, and after 15 min [^{3}H]ACh (3.2 μM) and Ringer are added to achieve the desired concentration in a total volume of 8 ml. This gives 30 nM AChR and up to 180 nM [^{3}H]ACh. Half saturation occurs at 7 to 10 nM ACh. From each tube 0.5 ml is withdrawn and counted in 6 ml of Biofluor to give the total counts. The remainder is centrifuged at 39,000 rpm for 90 min. If volatile anesthetics have been used aliquots are taken for GC analysis immediately on opening. Supernatant, 0.5 ml, is taken for the free counts.

Filtration assays are carried out similarly in principle. Filtration is performed on glass fiber filters and the filter correction is included in that obtained for nonspecific binding, using toxin preincubated receptor.

The effects of a wide range of general anesthetics on equilibrium binding of [^{3}H]ACh has been studied. In order to survey the anesthetics an assay is set up where about half of the receptors are occupied by [^{3}H]ACh. When increasing concentrations of anesthetic are added, increased or decreased binding may be readily detected. Suitable concentrations of selected anesthetics may then be chosen and full ACh binding curves made to define the cause of changes in binding.

All the volatile anesthetics increase the binding of [^{3}H]ACh at low concentrations. For halothane, chloroform, and diethylether this effect is first detectable at concentrations that anesthetize tadpoles at room temperature (the temperature of the binding assay). Maximum binding occurs at about five times the anesthetic concentration. At higher concentrations binding decreases, eventually in an irreversible manner. This pattern is also

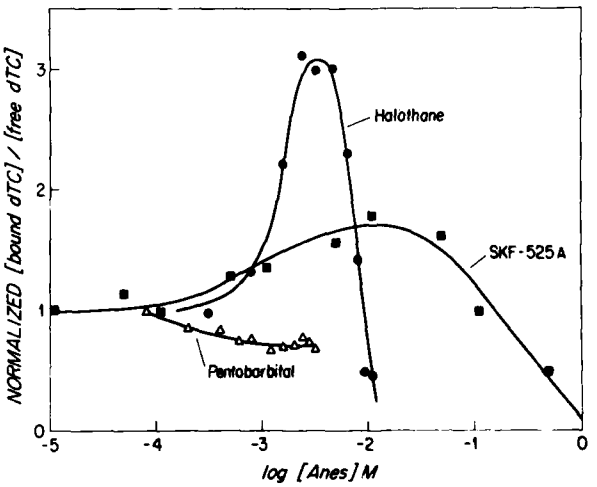

FIG. 1. A comparison of the effects of various concentrations of halothane, pentobarbital, and SKF 525a on the high-affinity equilibrium binding of [^{3}H]*d*-tubocurarine to ACh receptor-rich membranes from *Torpedo californica*. The concentration of SKF-525a has been multiplied by 10^3 to avoid compressing the scale.

seen in the alcohols (ethanol, butanol, and octanol). It resembles that reported (4) for local anesthetics but occurs over a narrower concentration range. The barbiturate and amine anesthetics, on the other hand, decrease ligand binding and effects are detectable well within the range causing general anesthesia. The effects on the binding of the antagonist, *d*-tubocurarine, are very similar. These results are summarized in Fig. 1.

The binding of [^{3}H]ACh to the receptor does not follow the law of mass action. Thus in one experiment half saturation occurred at 8.5 nM and Hill analysis yields a dissociation constant of 20 nM and a Hill coefficient of 1.4. Binding curves with fixed concentrations of halothane or octanol, up to those causing maximum increase in binding, showed a progressive decrease in dissociation constant and no change in the Hill coefficient. With pentobarbital, on the other hand, the shift was to a higher dissociation constant, again without change in Hill coefficient.

The high-affinity binding of [^{3}H]*d*-tubocurarine, which saturates half the ACh sites, gave mass action binding with a dissociation constant of 30 nM. Halothane and pentobarbital acted, as with ACh, by changing the receptor's affinity (Fig. 2).

These data suggested that the action of both halothane and pentobarbital could not be exerted by a lipid perturbation mechanism. However, in certain lipid bilayers pentobarbital orders the lipids. Accordingly a small amount of the spin label 5-doxyl stearic acid was incorporated into the membranes. Results of this electron spin resonance (ESR) study showed that both agents disordered the lipid (Pringle and Miller, *unpublished data*). Thus it was unlikely that lipids could be involved in the actions of both agents.

EQUILIBRIUM BINDING OF ACETYLCHOLINE AT PRESSURE

To see if pressure could be antagonizing the effects of either pentobarbital or halothane, filtration experiments were carried out in an 8-inch diameter steel chamber. Pressurization is achieved with the nonanesthetic gas

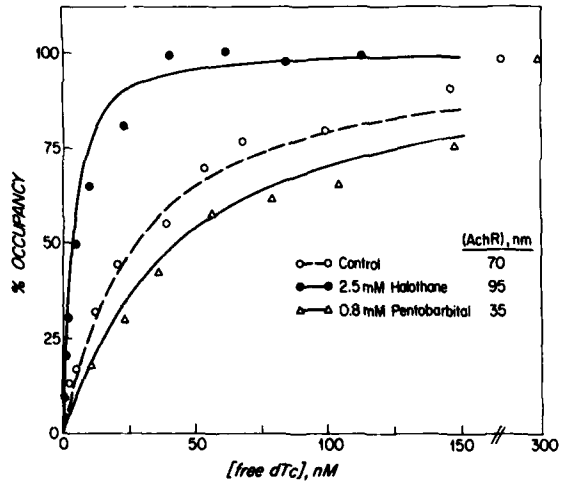

FIG. 2. Effect of fixed concentrations of halothane (2.5 mM) and pentobarbital (0.8 mM) on the equilibrium binding curve of [^{3}H]*d*-tubocurarine. Only α-bungarotoxin-displaceable binding is shown, and the *y*-axis has been normalized, since the receptor concentration was different in each experiment.

helium. Twelve filtration units are mounted on a turntable in the chamber. They may be moved in turn to a position where they may be remotely connected to the outside of the chamber. This process is operated and timed by a microprocessor so as to give smooth, reproducible filtering. Solutions to be filtered may be placed above the filters on diaphragms that rupture at the commencement of filtration, or may be delivered from motor-driven syringes.

Helium pressure decreases both [^{3}H]ACh and [^{3}H]*d*-tubocurarine binding. The effects of anesthetics are the same at high pressure. Halothane and octanol both increase binding equally at 5 and 300 atm, whereas pentobarbital decreases it equally (all relative to control binding at the same pressure).

Binding curves show that pressure changes only the Hill dissociation constant. Halothane is too volatile for work of the highest precision, and octanol was therefore used instead for detailed studies. Pressure causes a similar shift in the dissociation constant in the presence and absence of a fixed concentration of octanol. Since the effects are additive and act only on the dissociation constant, it is possible to obtain a binding curve in the presence of both octanol and 300 atm pressure, which is experimentally indistinguishable from that at 5 atm without octanol. In one experiment the Hill dissociation constants were as follows: in 1 mM octanol at 5 atm, 6 nM; in 1 mM octanol at 300 atm, 16 nM; in the absence of anesthetic at 5 atm, 15 nM; and at 300 atm, 23 nM. Thus, in this respect pressure may reverse the effects of volatile anesthetics, whereas it acts additively with barbiturates. This situation is consistent with the view that volatile anesthetics are acting by a lipid perturbation mechanism, but barbiturates are not. Studies of ACh binding kinetics, however, demonstrate that the situation is more complex than this.

EFFECTS ON ACETYLCHOLINE BINDING KINETICS

To elucidate further the above changes, kinetic studies have been performed. A simple classic mixing device was built. Two syringes, one containing receptor suspension (with or without anesthetic) and the other [^{3}H]ACh, are driven equally by hand with a rigidly

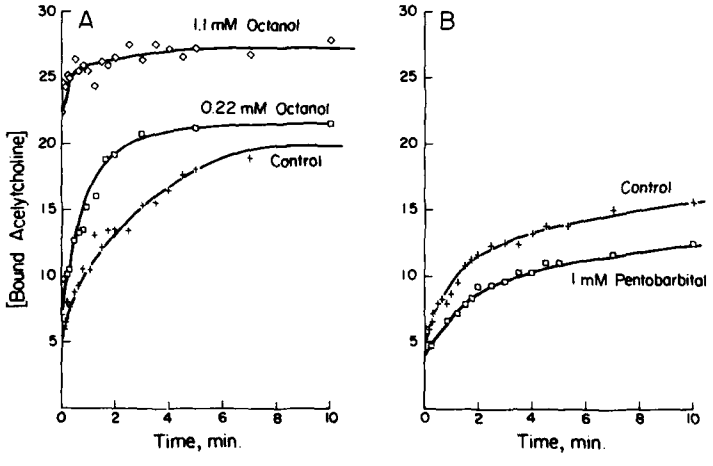

FIG. 3. Kinetics of [^{3}H]ACh binding to ACh receptor-rich membranes. The membranes were preincubated with anesthetic where appropriate and then rapidly mixed with the ligand and filtered at the times shown. Initial conditions: **left,** 50 nM ACh and 25 nM receptor; **right,** 25 nM ACh and receptor.

mounted drive block. Their contents pass through a T-mixer into an aging syringe. After a suitable interval this aliquot is discharged onto a Millipore filtration device. The drive syringes may be refilled from reservoir syringes connected by valves and all tubes are cleaned before the next mixing event. These studies are based on the classic work of Cohen, outlined in an earlier chapter of this volume.

Figure 3 shows the results for a set of experiments. The kinetics are biphasic. At the lowest concentration binding occurs too rapidly to be detected by our technique, whereas at higher concentrations this fast phase is followed by a slow phase with a half-life of approximately 1 min. Log-linear plots (not shown) clearly reveal the biphasic nature of these curves. It can be seen that octanol increases the amplitude of the initial fast phase of binding whereas pentobarbital has the opposite effect, although less dramatically. These data are thus consistent with the equilibrium binding data.

We have not developed equipment specifically to study kinetics in our pressure chamber. However, the syringes normally used for delivery of solutions to the filters can be employed for experiments in which the mixing time is of the order of seconds. Such experiments show that neither in the presence nor the absence of anesthetic does pressure have a large effect on the amplitude of the fast phase. Thus it seems probable at this level that anesthetics and pressure may not interact directly; indeed they may act at independent sites.

Our data are too preliminary for detailed interpretation. However, within the cyclic scheme of Katz and Thesleff (9), elaborated on in some detail by Cohen earlier in this volume, the following seems clear. Octanol and pentobarbital perturb the preexisting equilibrium between high- and low-affinity forms of the receptor in opposite directions. Pressure, on the other hand, has little effect on this equilibrium. One may tentatively conclude then that the effects of these perturbers on this equilibrium are independent of lipid fluidity, at least in the concentration range studied. Pressure probably slows the rapid high-affinity binding of ACh, since the overall dissociation constant is lowered, but detailed interpretation of the pressure work must await more adequate techniques.

Since the sum of the free energy changes in the cycle must be zero, changes at one point must be balanced elsewhere. We might expect, therefore, that if these studies were extended to the other quadrants of the cycle further effects of anesthetics might be found. Thus we are studying the effects of pressure and anesthetics on the agonist-elicited permeability response of membrane vesicles.

AGONIST-STIMULATED CATION EFFLUX

Although the latter studies offer plenty of ammunition to those who have the conviction that lipids are not involved in anesthetic action, they were carried out on the high-affinity binding of ACh, which is not directly related to the physiological function of the receptor. Numerous studies attest to the ability of anesthetics to block or shorten the action of electrically and chemically stimulated ion channels (1,5). A few studies show that pressure tends to prolong the action of such channels (6). We here present data on the action of anesthetics on a cholinergically stimulated ionophore.

It has been shown by a number of workers that the receptor-rich membranes from *Torpedo* form sealed vesicles (15). If these are preincubated with radioactive cations the ability of agonists to stimulate the release of these ions may be studied. One practical problem we faced was the complexity of published techniques. In particular, extensive washing of filters was involved to remove the cations external to the vesicles, followed by counting of the filters to determine the cat-

ions remaining in the vesicles. The external ions involved were largely those remaining from the loading procedure, together with those released by agonists. In the pressure chamber washing of the filters would be difficult. We solved this problem by using exclusion chromatography to separate the vesicles from the external ions. We were thus able to determine the agonist-stimulated cation efflux by assaying the filtrate directly and subtracting the appropriate controls to correct for background efflux.

High concentrations of carbachol were found to release essentially all the ions within 20 sec at 4°C. At lower concentrations longer times were required (Fig. 4). The equilibrium cation release was concentration-dependent; the data could be fitted to a Hill plot and yielded a dissociation constant of 1.5 μM and a Hill coefficient of 1.8.

The effects of general anesthetics on the maximum flux at 20 sec were examined. All the general anesthetics decreased the flux in a concentration-dependent manner, causing complete block at higher concentrations (Fig. 4). The nonanesthetic anticonvulsant, phenytoin, did not block the flux, even though it perturbed ACh binding. Thus in general the pattern of pharmacological effects paralleled that seen in general anesthesia, as might be expected from the extensive electrophysiological work at the neuromuscular junction (10,11,12,21).

The interaction with anesthetics is quite complex. Even with 10^{-3}M carbachol, when the control flux becomes maximal within 20 sec, in the presence of pentobarbital flux is prolonged. That is, channel opening is still occurring. This may also be true of the controls, but when the vesicles are depleted of labeled cation further channel opening cannot be detected. The time scale of the response indicates that effects on both the channel and on desensitization (conversion to the high-affinity form of the receptor) are involved. Comparison with the data for ACh

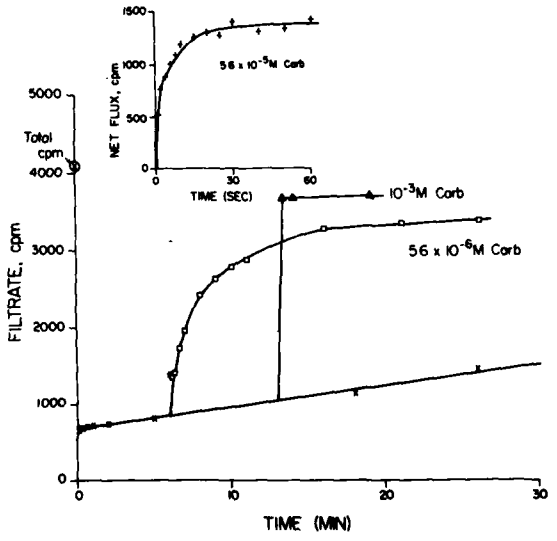

FIG. 4. Characterization of $^{86}Rb^+$ efflux from cholinergic vesicles. X, represents the background or passive leak; zero time is 10 to 15 min after elution from the exclusion column (see text). At various times aliquots of vesicles are added to carbachol to yield the final concentrations shown. Inset, +, shows net flux for one such addition, where net flux is the difference between stimulated and background efflux. X, represents the total counts in each aliquot.

binding suggests that at the earliest times we can assay little desensitization has occurred. However, at the present time a detailed interpretation must await the results of continuing work. The essential point is that there is no relationship between effects of anesthetics on cation flux and on equilibrium agonist binding.

CONCLUSIONS

We have demonstrated that the actions of general anesthetics in a single postsynaptic membrane are complex. The volatile and intravenous agents have opposing effects on the equilibrium binding of cholingeric ligands to their receptor. Volatile agents all displace the equilibrium between high- and low-affinity conformers of the receptor toward the high-affinity form, whereas other agents have the opposite effect. Although pressure decreases the equilibrium binding it has little effect on high-low affinity conformer equilibrium. Thus these actions have no parallel with anesthetic potency and are inconsistent with the lipid hypothesis. Several mechanisms could well be at work (see, e.g., chapter by Cohen, *this volume*.)

On the other hand, all general anesthetics, but not a nonanesthetic anticonvulsant, decrease the cholinergically stimulated cation efflux from vesicles and this action is opposed by pressure. The pattern of action is thus consistent with physiological actions of anesthetics and is also consistent with the lipid hypothesis. Although the actual mechanism remains to be demonstrated, it is interesting to note that gross perturbations of their lipid can effect stimulated efflux from vesicles (2).

Although one could speculate further about the mechanisms involved it seems unprofitable to do so at present. One advantage of this system is that it should prove possible to experimentally test the many hypotheses surrounding the mystery of anesthetic action. The complexities encountered in this single synapse suggest that extrapolation to other synapses should be approached cautiously!

ACKNOWLEDGMENTS

K.W.M. is a recipient of a Research Career Development Award of the National Institute of General Medical Sciences (GM-00199). J.F.S. is a Fellow of the Swiss National Fund for Scientific Research. This work was supported in part by a grant from the National Institute for General Medical Sciences (GM-15904) to the Harvard Anaesthesia Center, and in part by joint funding from the Office of Naval Research and the Naval Medical Research and Development Command through Office of Naval Research contract N00014-75-6-0727.

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Undersea Biomedical Research Vol. 7, No. 4, December 1980

An apparatus for performing filtration assays in hyperbaric atmospheres

J. F. SAUTER, P. G. WANKOWICZ, and K. W. MILLER

Departments of Anaesthesia and Pharmacology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Sauter JF, Wankowicz PG, Miller KW. An apparatus for performing filtration assays in hyperbaric atmospheres. Undersea Biomed Res 1980: 7(4):257–263.—A detailed understanding of the effects of diving gases on the central nervous system will require extensive neurochemical studies. One standard tool of the neurochemist is the filtration assay used to measure the binding of neuroeffectors to receptors, for example. We describe here a system for carrying our 12 such assays in hyperbaric gaseous environments. The device fits in a small pressure chamber and consists of a syringe drive for delivering solutions to the filter units brought in turn under an injection nozzle by a conveying carousel. A modified commercial filter apparatus is held on top of a vacuum-tight collection compartment. Solution is injected into an incubation well above the filter. After stirring and incubation, filtration is performed by allowing the positive pressure of the chamber to burst a diaphragm at the bottom of the incubation well and push the solution through the filter. The binding of [^{3}H]acetylcholine to receptor-rich membranes from *Torpedo californica* was studied. In one experiment a percentage receptor occupancy of 49.5 ± 1.4 at 5 atm was reduced to 41.7 ± 0.9 at 300 atm.

hyperbaric filtration apparatus
gas pressure
filtration assay

To obtain a complete understanding of the physiological effects of pressure and of diving gases will require a detailed approach in which the effects on intact animals are compared to the results from electrophysiological and neurochemical studies on isolated systems. Few neurochemical studies have been reported under high pressure of gases (1). One common tool used in neurochemical studies is the filtration assay, which may be used, for example, to measure binding of neuroeffe tors to their receptors. In this note we describe a filtration apparatus for use in gaseous hyperbaric environments, and we demonstrate that the binding of [^{3}H]acetylcholine to its receptor may be measured with high precision in a small chamber pressurized to as much as 300 atm with helium.

GENERAL DESCRIPTION OF APPARATUS

Our overall objective was to design a system capable of achieving a dozen filtration assays during a given pressure exposure. We also needed to mix accurately known volumes of

various solutions in situ and stir them before filtration. The solution had to be achieved within the confines of an existing pressure chamber (2) that provided a cylindrical working space of 18 cm i.d. by 61 cm.

A general view of the final apparatus is shown in Fig. 1. It consists of three parts: a syringe drive *(left)* for delivering solutions; a mixing and filtration carousel with 12 movable filtration units *(right)*; and a system for applying suction to the filter (not shown, but see Fig. 3).

The syringe drive subsystem is conventional and is not described in detail. Because of the space limits in our chamber this unit was custom made, but any commercial system (e.g., Harvard pump, Harvard Apparatus, Millis, MA; or Hamilton Microlab P, Whittier, CA) could be substituted. Two features of our system are of sufficient interest to deserve attention. Stepping motors, working on a threaded lead principle, were folded back on the syringe so that they pull, rather than push, the piston. This arrangement saves space. The system can deliver a series of small aliquots from a large syringe (typically 1.5 ml from a 50-ml syringe) with a precision better than 1%, as determined both on the bench and in the chamber by weighing aliquots of water delivered from a syringe. Plexiglas mounting plates were used to allow the operator an unobstructed view through the unit.

Filtration units

The filtration unit had to allow the solutions to be mixed, incubated, and subsequently filtered. Standard Millipore or Whatman glass fiber filters were to be used, and for simplicity these were to be held in a suitably modified and commercially available filter unit. The design now in use is illustrated in Fig. 2. It consists, from top to bottom, of four functional units: 1) an incubation well, 2) a breakable diaphragm, 3) a filter holder, and 4) collection vessel. The whole unit disassembles to allow both retrieval of filter and filtrate after the experiment and

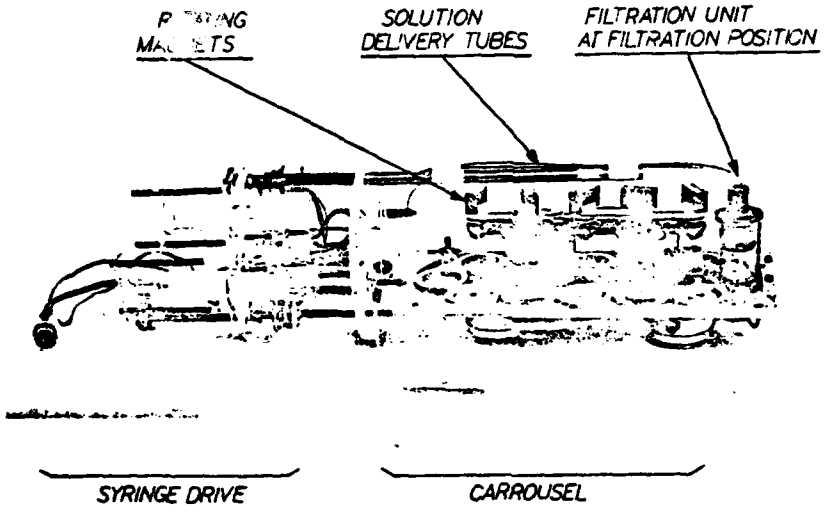

Fig. 1. View of the complete filtration system, showing the syringe drive with 2 of 4 syringes mounted, carousel with 3 of 12 filter units, and 5 rotatable bar magnets on top. Rings on the carousel are spacers used only in experiments with less than 12 filters.

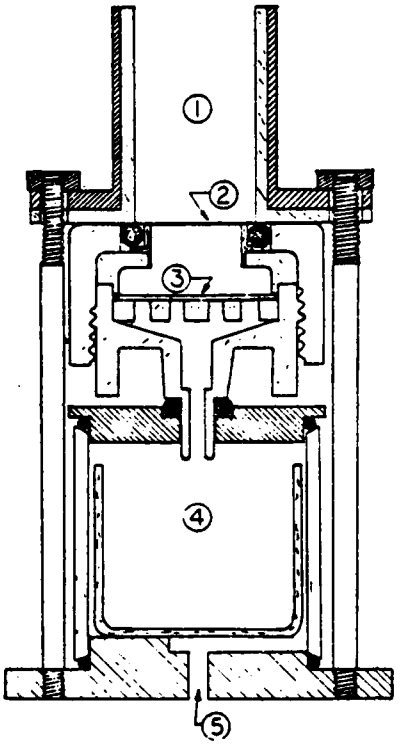

Fig. 2. Section of a filtration unit: 1, incubation well; 2, diaphragm; 3, filter; 4, collection vessel; 5, gas evacuation vent.

decontamination and washing before reuse. As many as12 such units may be accommodated on the carousel.

The incubation well is a Delrin-lined cylinder (1.5 cm i.d. by 3 cm) open at the top and closed at the bottom with a burstable plastic diaphragm (polyvinylchloride, 10–20 μm thick. Sandwich Bag, Purity Supreme, Boston, MA). Solutions may be added to this from the syringe drive subsystem and mixed by a Teflon-coated, magnetically driven stir bar. The filter holder is a Nuclepore (Pleasanton, CA) in-line type, 25 mm, modified externally to seat between the diaphragm and collection vessels on O-rings. The filtrate drains into the lower Plexiglas-walled chamber, where it is collected in a 5-ml glass vessel. A central hole in the base of the unit provides a pressure vent.

Filtration carousel

Twelve filtration units can be positioned in the carousel's raceway. They may be driven around the raceway by two motor-driven capstans driving a compressible O-ring belt (Buna N, 70 durometer). The belt constitutes the inner race and drives the filter units by rotating them against the outer stationary race. The central division also contains a variable speed 12-V DC motor linked by belt drive to five axially mounted horizontal bar magnets, which may be rotated to drive the stirrers in the incubation wells.

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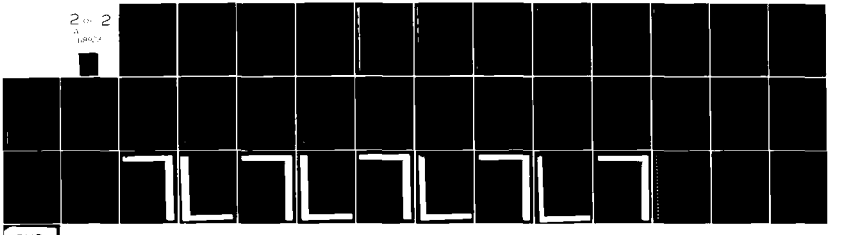

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Filling of the incubation wells and filtration are both performed at the end opposite the syringe drive, where the procedure may readily be observed through a chamber window.

Suction system

Suction for filtration is achieved by using the difference between the chamber pressure and room pressure. Two devices are required—the first to lock the base of the filtration units to a pipe venting through the chamber wall (Fig. 3), the second to control the pressure differential between the pipe and ambient pressure (Fig. 4).

The filtration lock (Fig. 3) is situated under the carousel at the window end position (Fig. 1, *right*). It is activated by opening of the piston vent pipe (Fig. 3, A) to low pressure, thus automatically raising the differential piston, D, to lock the O-ring to the base of a filtration unit at B. As the pressure in the differential piston continues to fall the spring-loaded check valve, E, opens; the subsequent evacuation of gas from the filter unit results in rupture of the diaphragm (Fig. 2), and the contents of the incubation well then fall onto, and pass through, the filter. Close control (see below) of this operation is required, particularly at the highest pressures, to avoid rupturing the filter. The design of the filter support screen is also important—large unsupported areas must be avoided. Control should be such as to avoid dispersing or aerosolizing the filtrate, both because this may lead to experimental anomalies and because the filtrate is radioactive.

Very smooth filtration, even at 300 atm, can be achieved by using the exhaust control unit shown schematically in Fig. 4. In normal operation valve A is open so that any minor leaks in the closed valves B and C do not activate the differential piston. To achieve filtration valve B is opened and then the pressure equalization valve (valve A) is closed. Valve C is controlled automatically because this enables achievement of the highest reproducibility, but manual control is satisfactory; we use a Kim-1 (Commodore, Santa Clara, CA) microprocessor for this, but any adjustable timing device or circuit would be suitable. Opening C allows venting to the room through the micrometer valve D and activates the filtration sequence described above. The setting of the micrometer valve is determined empirically, starting with a small setting and increasing the setting until the desired result is achieved. For this purpose it is very useful to be able to observe the filtration process through the chamber window. In practice two such valves are required to give a wide enough range of flows to achieve filtration from 2 atm to 300 atm. At any given pressure a range of micrometer settings will be found. At the lowest setting a slow filtration sequence, requiring about 2 s to break the diaphragm and 5 s to filter, yields very smooth filtering. At the highest setting almost instantaneous filtration occurs, but gas flow must be stopped quickly to prevent splattering the contents of the collection vessel. After filtration, B is closed, and A is opened to ensure the return of the suction piston to its lower position. Because the filtrate is radioactive it is important to effectively baffle the exhaust pipe E to prevent aerosolized radioactivity from entering the laboratory during vigorous filtration. For this purpose we lead the exhaust pipe to the bottom of a plastic bottle loosely filled with absorbent paper.

One further parameter that is conveninet to fine tune is the dead volume between C and D. This space is initially at 1 atm when valve C is opened; the resulting pressure surge is ideally sufficient to raise the differential piston but not to open the check valve. The latter opens as pressure falls under control of needle valve D. We adopted this pneumatic method because of space limitations, but if these are not critical, electrical elevation of a simpler lockup for delivering suction to the filter could well be satisfactory.

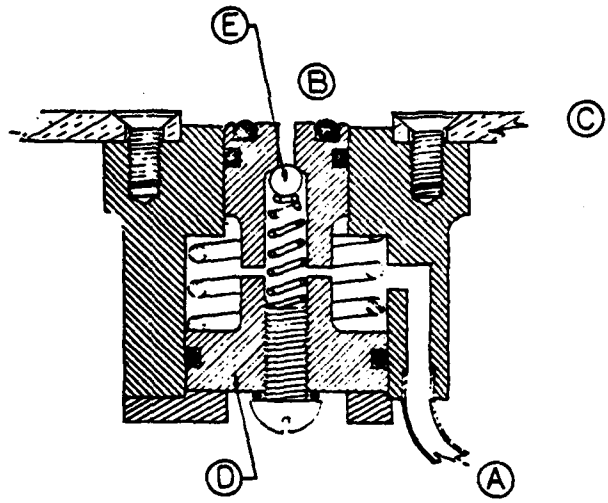

Fig. 3. Cross section of the lock that connects filtration units to suction during filtration. A, piston vent pipe connecting to controls (see Fig. 4) outside the chamber; B, top of differential piston that seals to the gas evacuation vent on the bottom of a filtration unit; C, level of the carousel table; D, differential piston that moves upward upon evacuation through A; E, spring-loaded check valve that opens after B locks to the filtration unit.

PROCEDURES AND RESULTS

The system used to test and develop our methodology was the binding of [^{3}H]acetylcholine to receptor-rich membranes isolated from the electroplaque of the ray *Torpedo californica*. Experimental procedures given here are only concerned with the high pressure aspect of the experiment. The techniques used are well established and details may be found elsewhere (3,

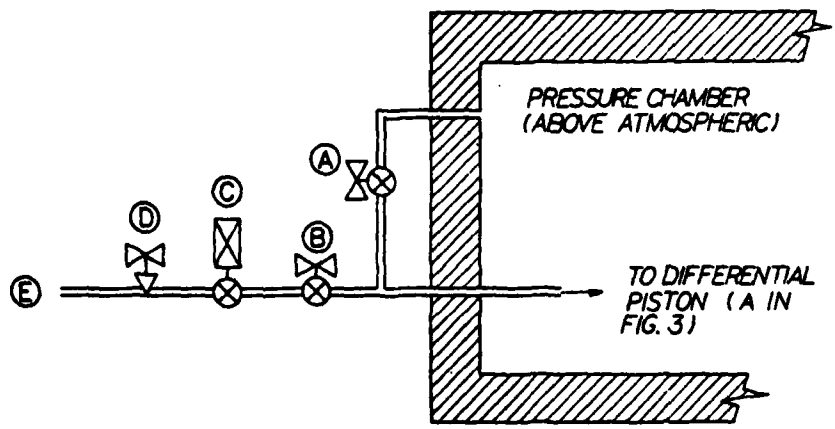

Fig. 4. Schematic of the external pneumatic arrangement used to control filtration. Valve C is electrically activated, valve D is a micrometer needle valve used to adjust the speed of filtration. System is duplicated for low pressure filtration (see text).

4). Briefly the principle of the experiment is that a suspension of acetylcholine receptor-containing membranes is mixed with an appproximately equal concentration of [^{3}H]acetylcholine. As the latter binds to its receptor its free concentration is reduced. After equilibration is complete, filtration allows determination of the remaining free [^{3}H]acetylcholine concentration in the filtrate after small corrections have been applied for nonspecific binding to the filter and to the biomembrane.

Three different ways of using our equipment were investigated. In the first two the solutions were added to the incubation well—either manually before the chamber was closed or automatically from the syringes at pressure. These two methods gave identical results. In the third method a stream of mixed suspension was delivered directly onto the filter in the absence of any diaphragm. Such a procedure would be necessary during experiments on the kinetics of binding, when the slightly variable time of diaphragm bursting is a disadvantage, or for equilibrium studies on the premixed solutions in the syringe when dispensing with the diaphragm is merely a convenience. All the experiments here were conducted at equilibrium. Initial experiments concentrated on reducing the standard deviations between filtrations. Initially such errors were in the range of 5% to 15%, but it was soon found that this percentage could be improved by removing the upper filter-holding grid, or screen, in the Nuclepore unit. The latter was designed to allow bidirectional flow and had been retained to avoid filter movement during compression. It is not shown in Fig. 2. With the screen removed the diaphragm method consistently gave standard deviations of about 2% in groups of four filtrations. Comparable accuracy could only be achieved in the absence of the diaphragms when suction was commenced a few seconds after delivery of the solution to the filter. Simultaneous suction and filtration at high pressure increased the standard deviation to 8%–10% at 300 atm.

The best standard deviation achieved above is comparable to what may be achieved by using conventional equipment on the bench. Such low variability suggested that no receptor-containing membranes were being forced through our filters. This conclusion was confirmed by refiltering on the bench filtrates collected from high pressure filtrations. During the early experiments catastrophic failure of the filters occurred sometimes, but development of the technique of applying suction, described above, has totally prevented this.

As an example of the sort of results that may be obtained, in one experiment in which the total receptor concentration in suspension was 35 nM and the total [^{3}H]acetylcholine concentration was 35 nM, we found the percentage of receptors occupied by acetylcholine to be 49.5 ± 1.4 (SD) at 5 atm helium, 49.0 ± 0.8 at 150 atm helium, and 41.7 ± 0.9 at 300 atm helium. Four filtrations were performed at each pressure.

This research was supported in part by an Office of Naval Research Contract (N00014-75-C-0727) and with funds provided by the Naval Medical Research and Development Command and in part by a grant from the Biomedical Research Support Grant of the N.I.H. at the Massachusetts General Hospital. J.F.S. was a fellow of the Swiss National Foundation for Scientific Research and K.W.M. is a Research Career Development Awardee of NIGMS (GM-00199).

Anyone desiring more detailed information on the equipment is invited to write to P.G.W. or K.W.M.—*Manuscript received for publication March 1980; revision received May 1980.*

Sauter JF, Wankowicz PG, Miller KW. Un dispositif pour l'exécution des essais de filtration dans les atmosphères hyperbares. Undersea Biomed Res 1980: 7(4):257–263.—Une comprehension détaillée des effets des gaz de plongeur sur le système nerveux central réclamera des études neurochimiques étendues. Un outil classique du neurochimiste est l'essai de filtration utilisé pour mésurer l'agrégation des effeteurs neuraux aux récepteurs par exemple. Nous décrivons ici un système pour mettre à exécution notre douze essais dans les environnements hyperbares gazeuses. Le dispositif s'adapte à une petite chambre de pression et consiste en une attaque-seringue pour délivrer des solutions aux unités filtres amenés en tour sous un ajutage d'injection par un carousel porteur. Un dispositif filtre commercial modifié est tenu sur le sommet d'un compartiment de

rassemblement vacuum-étanche. De la solution est injectée dans un puits d'incubation au-dessous du filtre. Après de l'agitation et de l'incubation, la filtration est exécutée permettant la pression positive de la chambre d' éclater un diaphragme sur le fond du puits d'incubation et pousse la solution à travers le filtre. L'agrégation des membranes riches de recepteurs [^{3}H]acétylcholine du *Torpedo californica* a été studiée. Dans une épreuve une occupation recepteur pourcentage de 49.5 ± 1.4 à 5 atm a été à 41.7 ± 0.9 à 300 atm.

dispositif de filtration hyperbare
prèssion de gaz
essai de filtration

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The Amelioration of the HPNS

Keith W. Miller

The object of this presentation is to examine in detail how far a pharmacological approach may be useful in the control of the HPNS. I shall not be much concerned with other important variables such as compression rate and temperature. The question posed in its simplest form is under given conditions how far do pharmacological agents alter the HPNS and what does this tell us about the underlying mechanisms? Given mankind's rather rudimentary knowledge of his own central nervous system only rather crude mechanistic models can be expected. This talk will cover three aspects of the topic. First, the use of gas-mixtures to ameliorate the HPNS. Here there is a simple model which accomodates the experimental data to a good approximation, makes some interesting predictions and leads to the conclusion that different aspects of the HPNS are mediated by different sites. Second, the latter rather non-specific approach can be replaced by one based on specific drugs. Here the aim is to selectively protect against those aspects of the HPNS which appeared above to have different sites of origin. In essence to perform a pharmacological dissection of the HPNS and to seek rationalizing models based on such data. This is an aspect of the subject which will be discussed by other speakers also. Third, I want to point out some directions for further study. In the CNS classical electrophysiological techniques are difficult to apply; the physiologist gravitates to the periphery. Biochemical techniques can thus fill a gap here. We have done some work which demonstrates the feasibility of doing such studies under hyperbaric conditions. The equipment we have developed should enable specific neurochemical questions to be approached.

All the work described here used mice as subjects. Experiments were carried out in a hyperbaric chamber under well controlled physiological conditions.

Gas mixtures were used to control the HPNS in the very earliest studies. The realization that helium pressure reversed the sedative effects of the anesthetic gas in these mixtures gave impetus to their application. Many of the findings are well known. We have systematically studied the effects of a number of anesthetic gases on different phases of the HPNS. All raise HPNS thresholds and their relative potency in doing so is roughly proportional to their anesthetic potency. When their ability to raise HPNS thresholds is compared on this basis, it is found that different aspects (for example chronic vs tonic convulsions) have their thresholds raised at different rates, suggesting different underlying mechanisms (Figure 1). This actually raises a difficult experimental problem. The end-points of the HPNS often occur quite close to each other on the pressure scale. When these end-points shift by different amounts the order they occur in may invert. It is often difficult to distinguish them. Thus work to better define end-points which occur at lower pressures is clearly important.

It is possible to rationalize most of these observations using the critical volume hypothesis. The purpose of doing so here is to emphasize some points that are not immediately obvious from the experiments. Empirically we observe that helium excites while argon is an anesthetic. Helium reverses argon anesthesia. Helium also acts very much like hydrostatic pressure. In the critical volume hypothesis this occurs because helium is so insoluble that little of it dissolves at the site of action; thus its expanding effect is small. So small in fact that the mechanical compression is large and net compression occurs. Argon is more soluble and

expansion occurs. The model equates compression with excitation and expansion with anesthesia. By mixing gases the two effects can be titrated ---- hence the use of trimix. But will helium always behave like pressure ? The model predicts not and is quite precise about when this happens. At a site where helium is more soluble than usual and where the compressibility is smaller than usual, helium is predicted to cause expansion. When one looks at the physical parameters of the sites we have characterized, the occurrence of sites which helium will expand seems very probable. There are indeed observations in the literature where helium interacts additively with nitrogen. The important conclusion is that we have no right to expect the trimix concept to work universally when helium is used as a pressure transmitter. Therefore for a given aspect of the HPNS which is of practical concern specific experiments should be performed to test the applicability of the trimix concept.

In the second part of this presentation we turn to the use of intravenous agents. We have compared intravenous anesthetics and non-sedative anticonvulsants as well as some other agents (Rowland-Jones, Wilson and Miller, unpublished data). The most striking contrast is seen between phenytoin and phenobarbital which have some structural similarities (Table 1). Our data show that all the end-points studied (tremors, spasms, clonic and tonic convulsions and death) have different pharmacological profiles. Furthermore, the percentage increase in the threshold is greater for some end-points than others.

The anesthetics urethane and phenobarbital gave excellent protection at high doses, in many cases doubling the threshold pressures (Table 1 and Figure 1). Urethane seemed particularly effective at preventing the tremors which showed an unusually low incidence with this agent. Phenobarbital was exceptional against

tonic convulsions and also raised the lethal threshold remarkably. Pressures of 250 Atm (8,250 fsw) were reached without modifying the compression profile (60 Atm/hr) compared to a control value of 130 Atm (4,300 fsw). In these experiments, as well as with trimix, there is a clear tendency for the tonic convulsion threshold to be elevated more rapidly than the lethal threshold. Consequently at high doses death intervenes before the tonic phase occurs, nicely illustrating that these effects have unrelated causes.

The non-sedative anti-convulsant, phenytoin, also illustrates the latter point (Table 1). No tonic phase was observed but death occurred at control pressures. Phenytoin was remarkable for its ability to potentiate tremors and spasms, an observation confirmed independently. It was the only agent to do so.

Other agents have been examined (Table 2). The overall picture is one in which only the anesthetic agents gave a broad protection; other agents gave selective protection, no protection or potentiation. The variety of responses to the non-sedative agents is encouraging. It justifies the systematic search for agents which may selectively protect against the early phases of the HPNS associated with diving. It is difficult to draw mechanistic conclusions from the latter studies. One must realize that many of the drugs effective against epilepsies, for example, have unknown modes of action. Furthermore, one must distinguish at least two types of anti-HPNS agent. The first acts on the primary site of pressure excitation and the second on the neural pathways which transmit the excitation to those areas mediating the behavioural response. By analogy with work on epilepsies several modes of action may be possible in each case. Thus it is much harder to draw conclusions about the specific, than the non-specific, agents. Only detailed studies on simpler systems can be expected to yield useful mechanistic information.

We have tackled the problem of extending HPNS studies to the neurochemical level by developing a filtration apparatus which will enable many of the *in vitro* CNS preparations to be studied in hyperbaric gases. Presently we are using this to study the properties of the nicotinic acetylcholine receptor from electric fish (a rich source), but the technique will allow one to pose a series of neuro-chemical questions. At present we have studied the effect of helium pressure on the binding of [^{3}H]-acetylcholine to its receptor. We find that the binding affinity is decreased without changing the number of sites or the cooperativity of binding. Volatile anesthetics have the opposite effect on binding. Thus the effects of helium and volatile anesthetics oppose each other. Preliminary studies show that other inert gases, such as argon and nitrous oxide, act in the same direction as volatile anesthetics. Thus, while the experimental problems should not be underestimated, it is quite feasible to carry out quantitive neuro-chemical experiments under hyperbaric conditions. One is thus in a position where precise questions may be posed and answered. Since the problem remains so ill-defined, however, and the *in vivo* neuropharmacology of the HPNS is scarcely charted, the choice of initial questions is difficult to define on a rational basis.

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TABLE 1 Comparison between nitrogen and some drugs (60 atm/hr)

Drug (xAD_{50})	Dose mg/kg	Coarse Tremor	Complete Spasm	Tonic Convulsion	Death
Nitrogen					
(0.21)	7.5 atm	ND			+ 28 atm
(1.4)	49 atm	ND		none	90 atm
Urethane					
(0.24)	230	+ 20			
(1.55)	1500	+ 63	+77	+ 100	+ 80 atm
Phenobarbital					
(0.12)	21	+ 24	+ 21		
(1.42)	160	+ 29		none	> 96 atm
Diphenylhydantoin	47	- 31		none	
Chlorpromazine	15 - 60				
Helium	50 ± 5	83 ± 4	102 ± 9		129 ± 10 atm

Note: ND = Not determined

Only changes in threshold 20 Atms are reported here

TABLE 2 Effects of 3 non-anesthetics on HPNS (200 Atm/hr)

Drug	Dose mg/kg	Coarse Tremor	Complete Spasm	Tonic Convulsion	Death
Helium		36 ± 7	36 ± 7	101 ± 9	117 ± 12 atm
Diazepam	2.5 2.0		+ 23	+ 26	+ 45 atm
Trimethadione	680				+ 40 atm
T.H.C.	60 - 120				

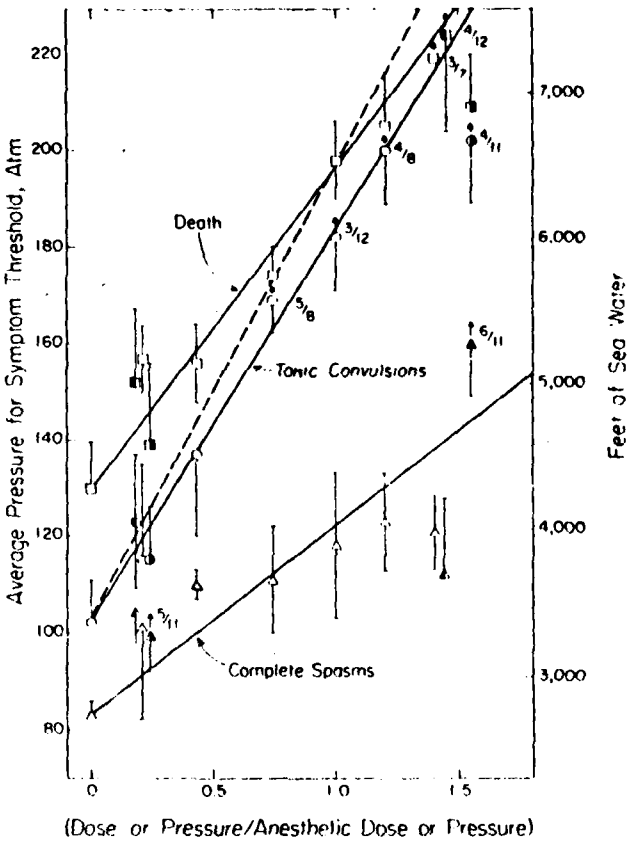

Figure 1:

The effect of nitrogen and two intravenous anesthetics on three HPNS end-points. Experiments were carried out on mice confined in a pressure chamber with excellent environmental control. Compression was carried out with helium at 60 Atm/hr and a pO_2 of 1 Atm. The nitrogen was added at the beginning of the compression. Triangles: complete spasm threshold - rhythmical tensing of the whole body which precedes clonic (Type I) convulsions. Circles: tonic (Type II) convulsions. Squares: pressure induced death. Open symbols are helium plus nitrogen. Symbols solid on the right are urethane and those solid on the left are phenobarbital. Error bars are standard deviations. Lines are drawn by eye. Fractions indicate when not all mice showed a given end-point. Vertical arrows indicate the value shown is a minimum because of the incomplete response in the latter cases. The effect of the anesthetic is greater on tonic convulsion thresholds. The dotted line suggests that the tonic convulsion threshold is raised above the lethal limit.

Undersea Biomedical Research, Vol. 8, No. 1, March 1981

Pharmacological evidence for multiple sites of action of pressure in mice

P. ROWLAND-JAMES†, M. W. WILSON, and K. W. MILLER

Departments of Anaesthesia and Pharmacology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Rowland-James P, Wilson MW, Miller KW. Pharmacological evidence for multiple sites of action of pressure in mice. Undersea Biomed Res 1981; 8(1):1–11.—The ability of eight diverse pharmacological agents to ameliorate the high pressure nervous syndrome (HPNS) in mice was studied. Data were obtained for the end points: coarse tremors, complete spasms, clonic convulsions, tonic convulsions, and death. The three anesthetics examined (nitrogen, urethane, and phenobarbital) gave good protection against all end points but especially against tonic convulsions. Furthermore, marked increases (>90 atm) were recorded in the lethal pressure in spite of a fixed linear compression. Some detailed differences among the anesthetics were also noted. Of the anticonvulsants, phenytoin protected against tonic convulsions but actually exacerbated some other end points. Diazepam gave some protection against all phases except the tremors, as did trimethadione. Tetrahydrocannabinol and chlorpromazine had little effect. The pharmacological profiles of these end points are all different, suggesting they represent the effects of pressure at separate and distinct sites in the central nervous system. The HPNS cannot be regarded as a single syndrome.

high pressure nervous syndrome
anesthetics
anticonvulsants

It has long been recognized that hydraulic compression of aquatic animals leads to a general stimulation of the central nervous system (for a review see Ref. 1). More recently it has been recognized that in amphibians the effects of hydrostatic pressure are similar to those observed when helium and neon are used as the compression medium (2). Mammals compressed hydraulically (3) show similar symptoms to those compressed in helium (4). Some of these effects of hydrostatic pressure have also been manifest in human oxyhelium deep diving (5), and they present a formidable obstacle to the extension of experimental dives to ever greater depths. In mammals this hyperexcitability, which has become known as the high pressure nervous syndrome (HPNS), is manifest in several stages ranging from fine tremors of the extremities, through clonic and tonic phases, to death.

The complexity of the HPNS experienced by mammals compressed in helium-oxygen has been established in a number of ways. One method that has proved useful is the pharmacologi-

†Pat Rowland-James died in 1977.

cal method. Thus, recently it was shown that type I (clonic) seizures in mice could be distinguished from type II (tonic) seizures by their differential sensitivity to phenytoin and reserpine pretreatment (6). These workers could also make the same distinction based on sensitivity to compression rate and other criteria. In an earlier paper the site of action at which inert gases cause anesthesia was distinguished from that at which they suppress hyperbaric convulsions (7). This conclusion was independently verified in a study on the action of barbiturates (8). In this paper we have examined a wide range of sedative and anticonvulsant agents given acutely to mice that were then compressed in helium-oxygen to pressures in excess of 250 atm (8250 fsw). Five different end points associated with the HPNS were observed. The pharmacological patterns found suggest that each phase of the HPNS is distinct from the others. Pharmacological strategies for controlling one phase may be ineffective against a subsequent phase at higher pressures.

METHODS

Male CD-1 mice (Charles River) weighing 20–30 g were used in all experiments. Hyperbaric experiments were performed in a 34-liter steel chamber rated to 300 atm and equipped with two 4-in. observation ports, a temperature controller and monitor, and moisture and carbon dioxide scrubbers. A more complete description has been given in an earlier paper (9).

Three groups of mice were exposed to pressure in each experiment. Two groups of either 5 mice (fast-compression series) or 4 mice (slow-compression series) were placed in individual cylindrical wire mesh cages in front of each window. The third group always consisted of 2 restrained mice with thermistor probes inserted rectally. Rectal temperature was maintained at 37°C ± 1°C by adjusting the chamber temperature, which had a fast response time. At pressure the chamber temperature was generally around 35°C.

Animals were injected with the drugs before each experiment, as reported under RESULTS. In the fast-compression series, 6 mice with drug and 4 with injection vehicle were observed in each experiment. This procedure was adopted in the slow-compression series only for chlorpromazine, because control data were simultaneously being accumulated in a set of experiments with helium, which also provided data for a separate series of experiments on gas mixtures. Fewer internal controls were therefore run for these intravenous agents, but in each experiment two different situations were present: either 4 controls and 4 drugged mice, or 4 mice with one drug and 4 with another. In this way the occasional group of mice with unusual HPNS susceptibility was readily detected.

Mice were observed continuously by two observers, or occasionally by one observer and a television monitor. The observer was always unaware of the treatment received by individual animals. The occurrence of the HPNS was based on visually observed behavior. The end points used were defined as follows: coarse tremors occurred when the fine tremoring of the limbs observed at lower pressures became generalized over the whole body; complete spasms were rhythmic tensing and relaxing of all muscle groups; clonic convulsions were when the animal lost its upright posture (for convenience, this end point, which occurs only 7 atm after the end point for complete spasms, was omitted in the fast-compression series); tonic convulsions involved contraction of muscles, extension of limbs, and cessation of breathing, often followed by a period of prostration.

Linear compressions were controlled manually at a rate of either 60 or 200 atm/h and were conducted with the use of helium. Compression was preceded by a 5-min period of oxygen flushing, yielding a partial pressure of 1 ATA. Incoming gas was mixed with the output from the gas scrubbing blower, and a further auxiliary fan was also employed to ensure thorough mixing.

Pressure thresholds for occurrence of HPNS were analyzed as means, plus or minus standard deviations, and tested for significance by Student's t test. In some cases pressure response curves were analyzed by the method of Waud (10) for quantal responses. The two methods of analysis gave similar results. The former method was adopted here, since it is consistent with that used by previous workers (6). In addition, the number of animals responding (r) and the number observed (n) are reported, because in some cases the incidence of a particular end point was reduced, sometimes even to zero. In these cases, the means given are calculated from the data for r animals, and represent lower limits. In addition to the mean threshold pressure and the actual incidence, the severity, or amplitude, of the HPNS might also be influenced by a drug, without alteration of either of the other parameters. Such cases are noted under RESULTS, although they necessarily involve a subjective judgment.

RESULTS

Table 1 shows the pooled data from many experiments carried out with helium alone. None of the control data from individual experiments involving drugged mice differed significantly from these means. In Tables 2 and 3 the results of various treatments are reported as changes in pressure thresholds relative to the pooled control values in Table 1.

The errors given in Tables 2 and 3 are the standard deviations of the difference between drugged and undrugged groups of animals calculated according to Bevington (11). The errors in the last row of Table 1 were obtained similarly. A difference of <2 SD may be regarded as of little significance. We have not given P values here because we do not wish to overemphasize the changes in threshold as a measure of the drug effect. It is also important to take into account changes in the percentage of incidence and severity of the symptoms.

The effect of compression rate was only highly significant for coarse tremors and death. The absolute elevation in threshold upon decreasing compression rate 3.3-fold was only in the range of 10–15 atm, but in the case of tremors this is a 1.4-fold increase. Our results for tremor are consistent with those of two recent studies (12, 13), although the magnitude of our effect may be somewhat larger than expected from averaging over a wider range of rates. No other workers have used complete spasms as an end point, but in a separate series of experiments in another laboratory using a tenfold range of compression rates, one of us found no consistent effect (14). Our tonic convulsion end point corresponds to the type II seizures of Brauer et al. (6), who also reported little change. Because of technical limitations, few workers have studied the fatal pressure systematically. However, our data are broadly consistent with previous work (15).

TABLE 1
HPNS THRESHOLDS FOR COMPRESSION OF MICE IN $He\text{-}O_2$

Compression Rate, atm	Coarse Tremor, atm	Complete Spasm, atm	Clonic Convulsion, atm	Tonic Convulsion, atm	Death, atm
60	50 ± 5.1 60/60	83 ± 3.5 46/60	90 ± 7.4 47/60	102 ± 8.8 52/60	129 ± 9.7 45/60
200	36 ± 7.4 47/47	86 ± 7.4 44/47	— —	101 ± 9.0 47/47	117 ± 11.7 40/42
Difference	14 ± 9.0	−3 ± 8.2	—	1 ± 12.6	12 ± 15.2

TABLE 2

EFFECT OF DRUGS ON HPNS THRESHOLDS: COMPRESSION RATE 60 ATM/H

Drug (Dose/ED_{50})	Dose, mg/kg	Coarse Tremor		Complete Spasm		Clonic Convulsion		Tonic Convulsion		Death		Maximum Pressure (Survivors)
		r/n	ΔP	*r/n*	ΔP	*r/n*	ΔP	*r/n*	ΔP	*r/n*	ΔP	
Nitrogen												
(0.21)	7.5 atm	ND*		4/8	18±18	8/8	26±8	6/8	13±22	8/8	28±12	162 (0)
(0.74)	26 atm	ND		7/8	28±12	8/8	39±10	5/8	67±11	8/8	45±11	185 (0)
(1.0)	35 atm	ND		11/12	35±15	9/12	50±12	3/12	80±14	12/12	69±13	216 (0)
(1.2)	42 atm	ND		8/8	40±11	8/8	46±11	4/8	98±14	8/8	76±15	214 (0)
(1.4)	49 atm	ND		6/7	38±8	7/7	56±11	0/7	--	3/7	82±14	225 (4)
Urethane												
(0.24)	228	11/11	20±8	5/11	16±8	9/11	9±13	7/12	13±14	11/11	10±20	175 (0)
												149 (0)
												142 (0)
(1.55)	1500	7/11	63±26	6/11	77±12	11/11	86±15	4/11	100±16	10/11	80±15	213 (1)
												205 (0)
												225 (0)
Phenobarbital												
(0.19)	21	12/12	24±7	12/12	21±7	12/12	28±15	7/12	21±17	12/12	23±18	191 (0)
												154 (0)
												160 (0)
(1.42)	160	10/12	34±9	12/12	29±16	12/12	59±15	0/12	--	4/12	82±22	213 (3)
												226 (2)
												251 (3)
Phenytoin												
	47	12/12	-31±8	11/12	-25±17	12/12	-5±12	0/12	--	12/12	-6±18	135 (0)
												140 (0)
												147 (0)
Trimethadione	630	10/12	12±10	8/12	6±9	9/12	11±12	9/12	4±10	12/12	1±20	128 (0)
												145 (0)
												173 (0)
Chlorpromazine												
	15	3/4	-1±5	5/5	11±5	ND		10/12	2±14	7/11	-10±21	139 (3)
												134 (1)
	30	5/5	-2±5	10/11	1±13	ND		10/13	5±15	10/10	-15±13	127 (0)
	60	5/5	-5±11	9/10	2±14	ND		6/11	11±19	11/11	-17±18	137 (0)

*ND, not determined. ED_{50}, dose that anesthetizes 50% of mice at 1 atm (9). *r*, Number of animals responding; *n*, total number of animals. ΔP, change in HPNS threshold, in atm.

All animals exposed to helium exhibited tremors; 94% and 77% exhibited complete spasms at the fast and slow rates, respectively; 78% had clonic convulsions during the slow compression; and 100% and 87% had tonic convulsions. Part of the failure to observe end points in all animals may arise from the overlapping of phases of the HPNS; e.g., complete spasms, clonic convulsions, and tonic convulsions all occur within a range of some 20 atm. Consistent with this notion, tremors, which are well separated from other end points, were always observed. The incidence of death is less than 100%, because in some experiments insufficient pressure was available in the helium accumulators to maintain a linear compression rate, and these experiments were terminated at this point. The means reported are for the animals that died and do not include the survivors. Where there were survivors in more than one experiment, each maximum is given with the number of survivors in parentheses in Tables 2 and 3. Where there were no survivors, the maximum pressure was that at which the final mouse died.

TABLE 3

EFFECTS OF DRUGS ON HPNS THRESHOLDS: COMPRESSION RATE 200 ATM/H

Drug	Dose, mg/kg	Coarse Tremor		Complete Spasms		Tonic Convulsions		Death		Maximum Pressure, atm
		r/n	ΔP	*r/n*	ΔP	*r/n*	ΔP	*r/n*	ΔP	
Diazepam	2.5	6/6	6±8	6/6	11±10	6/6	12±12	5/6	18±13	156
	5	6/6	2±8	6/6	14±9	6/6	16±11	6/6	22±18	156
	10	12/12	3±8	12/12	15±11	12/12	22±13	10/12	26±15	163
	20	6/6	5±10	6/6	23±8	6/6	26±14	4/6	45±15	175
Trimethadione	680	12/12	0±12	12/12	20±12	11/12	31±22	9/12	40±14	173
Tetrahydro-cannabinol	60	8/8	0±10	8/8	5±19	8/8	3±13	3/3	−9±20	123
	120	10/12	23±14	9/12	−4±13	11/12	5±13	11/12	−10±17	130

r, Number of animals responding; *n*, total number of animals. ΔP, change in HPNS threshold, in atm.

The effects of two long-acting general anesthetics, phenobarbital and urethane, were studied first in paired experiments at the slow-compression rate. The doses used may be compared to the ED_{50} doses for anesthesia, which are 113 and 970 mg/kg i.p. and rise linearly at the rate of 1.54 and 1.68 times per 100 atm, respectively (9). At the highest dose tested, phenobarbital and urethane gave good protection against all phases of the HPNS.

Urethane was injected about one hour before compression; an anesthetic dose gives a sleep time of about 4 h (9). Thus, above 180 atm one might expect its effects to be weakening. At the lowest dose, about one-fourth the anesthetic dose, urethane raised the tremor and spasm pressures by 40 and 19 atm, respectively, without causing marked changes in the other end points. At the highest dose the mean tremor threshold was 113 atm, but nearly half the group failed to exhibit tremors, and in those that did, the tremors disappeared after 10 min. They were then symptom-free until 6 of the 11 mice exhibited violent spasms at 160 atm, followed rapidly by clonic convulsions, tonic convulsions, and death at 209 atm. Thus, on the average, this dose doubled all the onset pressures except death, and the ability to reduce the severity of tremoring was exceptional.

Phenobarbital was injected s.c. some 3 h before compression to allow time for maximum protection to develop at our low, anticonvulsant dose (16). Anesthetic doses provide sleep times of more than 8 h (9). At the anticonvulsant dose, phenobarbital gave very similar results to urethane, but it tended to do better at postponing the later phases of the HPNS. At the high dose, phenobarbital attenuated the symptoms of the HPNS, but the incidence of tremors, spasms, and clonic convulsions remained high, and their threshold pressures were not improved by the increase in dose as much as with urethane. On the other hand, the tonic phase was completely suppressed and the lethal pressure raised markedly, only 4 out of 12 animals dying. In one experiment where the compression rate was maintained up to 251 atm, only one mouse died (at 232 atm).

Experiments with nitrogen could not be randomized satisfactorily. One experiment was performed at each partial pressure, except for 35 atm, where two experiments were performed. The nitrogen was added at the beginning of the compression, and compression continued with helium. Nitrogen itself caused fasciculation, and therefore no attempt was made to record tremors. Nitrogen gave dose-dependent protection against all the recorded phases of the HPNS (Fig. 1), but it was not as effective against complete spasms and tonic convulsions in low doses as were urethane and phenobarbital.

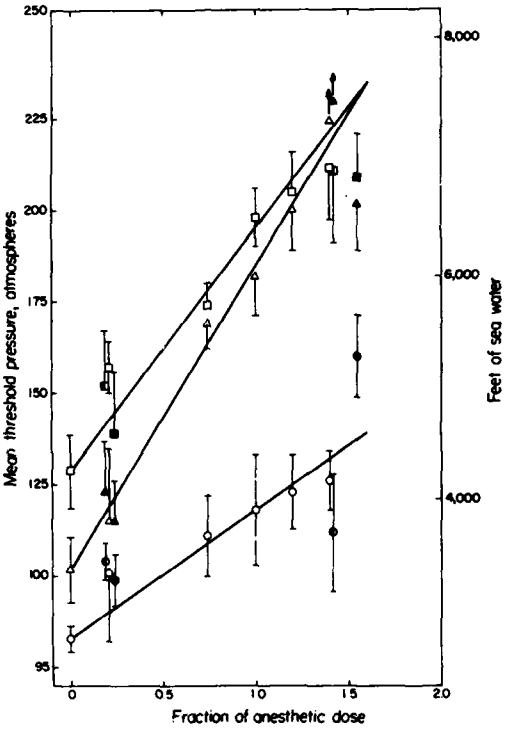

Fig. 1. The change in mean pressure in mice for first observation of complete spasms (*circles*), tonic convulsions (*triangles*), and death (*squares*), as a function of fraction of an anesthetic dose at 1 atm (9). *Open symbols*, helium and helium + nitrogen; *closed symbols*, urethane; *half-closed symbols*, phenobarbital. Data from Tables 1 and 2. Compression rate 60 atm/h. Oxygen partial pressure, 1 ATA. Rectal temperature, 37°C ± 1°C. The lines are least squares fits of nitrogen data constrained to pass through the helium controls, and the points were weighted by the number of animals responding. In some groups not all animals responded (See Tables).

Two nonanesthetic anticonvulsants were studied in paired experiments at their anticonvulsant doses. Both agents were injected subcutaneously (16). The duration of action of phenytoin was tested by challenge with 75 mg/kg intravenous pentylenetetrazole at 1.5, 3, and 4 h. All animals were protected against the tonic, but not the clonic, phase of the pentylenetetrazole seizure. Phenytoin dramatically potentiated the tremor and spasm phases of the HPNS, affecting both the severity and threshold pressure. It was ineffective against clonic convulsions (compare pentylenetetrazole) and death but abolished the tonic phase completely. Since the control tonic convulsion threshold is only 21 atm below the fatal pressure, this result may not be so dramatic as it appears. At subanesthetic doses, however, none of the other agents examined were even this effective, so that phenytoin's effect on the tonic convulsions must be regarded as exceptional, even if its magnitude remains uncertain. This potentiation of the early phase of the HPNS in mice is broadly consistent with the results of Brauer et al. (16), who used a dose of 60 mg/kg i.p. Phenytoin is also ineffective against hyperbaric convulsions in rats and guinea pigs (17).

Trimethadione gave only slight protection against any phase of the HPNS on slow compression but was more effective on rapid compression. At a lower dose (250 mg/kg i.p.) Brauer et

al. (6) reported a slowly developing protection against type I seizures, raising the threshold 20 atm, and a more rapidly occurring protection (25 atm) against type II seizures that declined with time. This difference from our slow-compression results is probably due to pharmacokinetics, because in our fast-compression series, where only 15–25 min elapsed from injection to start of compression, trimethadione gave good protection against all phases of the HPNS except tremor. Brain levels in mice are high for at least 30 min and fall by 40% at 1.5–2 h and by 60% at 3 h (18). Thus, our fast-compression data are almost certainly more representative of trimethadione's effect than are our slow-compression data.

Chlorpromazine was given intraperitoneally in aqueous solution or 9% sodium chloride. It made the animals lethargic and somewhat jumpy. These experiments were performed with the same protocol as the fast-compression series, except that compression was at 60 atm/h. In each experiment there were 10 mice; 2 controls with vehicles only, and 2 or 3 mice at each of the three doses. The jumpiness of the animals made the threshold of the coarse tremor difficult to define, and in some experiments no attempt was made to do so. The population thus appears lower in Table 2. In one experiment, compression was stopped before death occurred to check that chlorpromazine was still active as judged by the lethargy of the animals. Consequently, the population exposed to tonic convulsions appears greater than that exposed to death (Table 2). Pressure did not appear to alter the normally long half-life of chlorpromazine and its lack of consistent effect on the HPNS threshold, even at high doses, is thus real. It did, however, tend to lower the fatal pressure, and at the highest dose 5 mice died before reaching the pressure for tonic convulsion.

Diazepam was injected intravenously using an oil-lipid dispersion as vehicle (19). Controls (n = 20) injected with the vehicle did not differ from the pooled controls. No effect of diazepam on the tremor phase was seen, but the other three end points examined were all raised in a dose-dependent fashion without reducing incidence. Jeppsson and Ljunberg (19) found that at our lowest dose at least 50% of the mice were protected from electroshock between 12 and 58 min. At 10 mg/kg full protection was found for 45 min and 90% protection at 1 h. Our mean time from injection to start of compression was 20 min, so that spasms occurred at 50 min and death at 60 min post injection. Thus, only at our lower doses would one expect pharmacokinetics to reduce the effectiveness of the drugs.

Tetrahydrocannabinol was injected in propylene glycol-1% Tween in normal saline (1:9) after the method of Sofia et al. (20). These workers reported an ED_{50} against supramaximal electroshock seizures to be 44 mg/kg, while 120 mg/kg failed to protect against pentylenetetrazole-induced seizures. Protection was provided at 1 h after injection but had disappeared at 2 h. No marked effect on HPNS thresholds was seen except for tremors at high doses, when the animals were stuporous.

DISCUSSION

The extensive results of this work are summarized in simplified form in Table 4 in order that the patterns of pharmacological action upon the HPNS may more readily be discerned.

Our results enable the pharmacological dissection of the HPNS mentioned at the beginning of this paper to be taken a stage further. The high working pressure of our chamber has enabled us in addition to pharmacologically characterize the lethal threshold for the first time. Prior to this study, two conclusions were established in the literature. First, the site of general anesthetic action is distinct from that for clonic convulsions (7, 8), and second, the tonic and clonic phases are separable (6). Regarding the first point, in our present study only four agents have been tested against clonic convulsions. All the anesthetics except the low dose of urethane gave good protection, and the nonanesthetics gave no (phenytoin) or slight

TABLE 4
SUMMARY OF EFFECTS OF DRUGS ON THE HPNS

Drug (Dose/ED_{50}^{HP})	Dose, mg/kg	Compression Rate	Coarse Tremors	Complete Spasm	Clonic Convulsions	Tonic Convulsions	Death
Nitrogen							
(0.21)	7.5 atm	S[a]	ND	0[b]	+[c]	0	+
(1.4)	49 atm	S	ND	+	++[d]	+++[e]	+++
Urethane							
(0.24)	228	S	+	0	0	0	0
(1.55)	1500	S	++	++	+++	+++	+++
Phenobarbital							
(0.19)	21	S	+	+	+	+	+
(1.42)	160	S	+	+	++	+++	+++
Phenytoin							
	47	S	−[f]	−	0	+?+[g]	0
Chlorpromazine							
	15–60	S	0	0	0	0	0
Trimethadione							
	630	S	0	0	0	0	0
	680	F[h]	0	+	ND	+	++
Diazepam							
	2.5		0	0	ND	0	0
	20		0	+	ND	+	++
Tetrahydrocannabinol							
	120		+	0	ND	0	0

Changes in threshold pressure, atm. [a]S = slow compression. [b]0 = none. [c]+ = +20–40. [d]++ = +40–80. [e]+++ = >80. [f]− = −20–40. [g]+?+, See text. [h]F = fast compression.

(trimethadione) protection. Thus, these data do not distinguish strongly between anesthetic and anticlonic convulsive activity, although they are not consistent with previous conclusions. Our data do, however, confirm the distinction between clonic and tonic anticonvulsive activity (see below).

Thus our results are either consistent with or substantiate previous work. Below we systematically extend the pharmacological analysis to several other end points, considering first the relation of the highest pressure symptoms to each of the others.

Although the anesthetics studied here gave good protection against *death* (Fig. 1), we have previously shown (9) anesthetic doses of α-chloralose to provide no protection. In addition, the nonanesthetic trimethadione also gave fair protection. Thus, there is no unequivocal relation between anesthetic activity and ability to raise lethal pressure. The tonic phase, however, is protected better by a given dose of anesthetic than is the fatal phase. Figure 1 illustrates this point. The threshold pressures for death and tonic convulsions converge at 235 atm at 1.6 times the 1-atm anesthetic dose. Since tonic convulsions cannot be recorded after death, their percentage incidence also declines in a dose-dependent manner. The tonic convulsion line can only be fitted for the animals responding, and its slope is therefore only a lower estimate. Nonetheless, it is significantly steeper than that for death ($P < 0.001$). Furthermore, phenytoin prevented all tonic convulsions without altering the fatal threshold. Thus, the pharmacological distinction between the tonic and fatal phases of the HPNS is clearly established. Our data do not allow such a clear distinction to be made between activity against

clonic convulsions and death, but the dose dependence of nitrogen's protection against death is steeper than that for clonic convulsions ($P<0.001$) (Fig. 1 and Table 2). The small but consistent depression by chlorpromazine and the lack of effect of phenytoin on the lethal pressure both contrast with their action against complete spasms, as do the magnitude of the relative protective effects of phenobarbital and nitrogen. The tremor and lethal phases are distinguished by the actions of phenytoin and tetrahydrocannabinol. Thus, pharmacological profiles distinguish between hyperbaric death and all other phases of HPNS except for the clonic phase where only the dose dependency of protection differs.

In other investigations (6) the *tonic phase* has been distinguished from the clonic phase by a number of criteria. We found that phenytoin distinguished the tonic phase clearly from all other phases of the HPNS studied. In addition, the anesthetics were quantitatively more effective in protecting against the tonic than against either the complete spasm or clonic convulsion phases ($P<0.001$ in each case). (Compare slopes in Fig. 1.)

Phenytoin is the only agent that distinguishes the *clonic phase* from the spasm phase. This distinction is somewhat surprising, since to the observer the clonic convulsion appears as a continuation or culmination of the spasm phase. Phenytoin also distinguishes between the clonic phase and coarse tremors.

The *complete spasm phase* is distinguished from coarse tremors by diazepam, trimethadione, and tetrahydrocannabinol.

Thus, the pharmacological profiles presented both here and previously in this paper seem to suggest that each of the five phases of the HPNS observed are separable and distinct, with the possible exception of clonic convulsions and death. However, the significance of the above pharmacological dissection of the HPNS must be approached cautiously. A number of steps are undoubtedly involved in producing seizures. It is not possible at present to define these in any detail, but in general, two events can usefully be distinguished: the generation of a central focus of excitation, and the spread of this focus into other areas. Each of these events may be susceptible to pharmacological intervention at several levels (21). This uncertainty in the locus of action of the agents effective against the HPNS makes it difficult to draw mechanistic conclusions of a unitary nature. In general, the most lipid soluble drugs, the anesthetics, tend to do well against all phases of the HPNS. This suggests that these nonspecific agents can act at several sites, whereas the more specific agents probably act at fewer sites and are therefore capable of giving more precise but limited mechanistic information. Thus, the ineffectiveness of the tranquilizer chlorpromazine is noteworthy, since this agent blocks dopamine-sensitive cyclase in the brain (22), giving rise to Parkinson-like effects. Pressure also depresses dopamine-sensitive cyclase. This depression is opposed by anesthetics, and a relationship with the HPNS has been discussed (23). Our results suggest the situation is more complex.

Diazepam is known to bind a receptor in brain tissue, and the affinity of a series of benzodiazapines for this receptor correlate with their ability to antagonize pentylenetetrazole, but not convulsions induced by electric shock in mice (24). A recent report (25) showing that diazepam and phenytoin occupy the same receptor and that their affinities for it are modulated by γ-aminobutyric acid suggests the involvement of this axis in the tonic convulsions, since this is the only aspect of the HPNS that is alleviated by both agents. Furthermore, phenytoin protects only against the tonic phase of the pentylenetetrazole test and may even exacerbate the clonic phase (26). These examples suggest how the use of selective agents may provide further pharmacological insights into the HPNS.

From a practical point of view, the heterogeneity in the response of the different HPNS end points to the agents we have examined suggest two different pharmacological strategies for controlling the HPNS. The first is to use relatively nonspecific agents that depress all phases of excitability; the use of gas mixtures falls within this classification, for example. The second

is to seek drugs that are relatively selective for a given end point; such drugs might be extremely selective, as is phenytoin for tonic convulsions, or they might have a general efficacy combined with selective action against one phase. Urethane appears to be an example of the latter type; it protects against all phases but is relatively more potent against tremors than would be expected for a nonselective agent. Thus, the overall trend emphasized in Fig. 1 for the nonspecific agents should not obscure the fact that there may be significant differences between them that may usefully be exploited. This point has also been noted by other workers (27). The cellular basis for the observations is not clear, but since chemical anesthetics differ widely in their side effects, such differences, while remaining impossible to predict, should not be surprising.

Finally, our data on pressure-induced death represent the largest single systematic set of results for mice obtained under well-controlled conditions to date. They illustrate that pharmacological means alone can provide excellent protection. When the highest pressures achieved by the survivors are included, the mean lethal threshold is seen to be greater than 220, 209, and 224 atm for the highest doses of nitrogen, urethane, and phenobarbital, respectively. Thus, more than 90 atm of protection (69%) is provided without recourse to special compression rates (15, 28). Furthermore, the effect of 49 atm of nitrogen in the breathing mixture does not appear to influence the lethal pressure, since nitrogen protects against death equally as well as phenobarbital does at an equivalent anesthetic dose. Gas density thus appears not to be an important contributor to the lethal pressure in mice.

This work was supported jointly by the Office of Naval Research and the Naval Medical Research and Development Command through Office of Naval Research Contract N00014-75-C-0727. K.W.M. is a Research Career Development Awardee of the National Institute of General Medical Sciences (GM 00199). Reprint requests should be sent to Keith W. Miller.—*Manuscript received for publication April 1980; revision received August 1980.*

Rowland-James P, Wilson MW, Miller KW. Preuve pharmacologique des sites multiples démontrant l'action de pression sur les souris. Undersea Biomed Res 1981; 8(1):1–11.—Les capacités de huit agents pharmacologiques divers pour améliorer le syndrome nerveux à haute pression (SNHP) sur les souris ont été étudiées. Des données ont été obtenues pour les extrémités: trémulations aigues, spasmes complets, convulsions cloniques, convulsions toniques, et mort. Les trois anésthétiques observés (nitrogène, uréthane, et phénobarbitol) fournissaient une bonne protection contre toutes les extrémités, mais surtout contre les convulsions toniques. De plus, des accroissements marqués (>90 atm) ont été notés dans la pression mortelle malgré une compression linéaire fixe. Quelques différences détaillées parmi les anésthétiques ont aussi été notées. Le phenytoin protège contre les convulsions toniques, mais par contre aggravait d'autres extrémités. Le diazepam fournissait une protection adéquate contre toutes les phases, sauf les trémulations, ainsi que le triméthadione. Le tétrahydrocannabinol et le chlorpromazine ont eu peu d'effet. Les profils pharmacologiques de ces extrémités diffèrent tous, ce qui suggère qu'ils représentent les effets de la pression en des sites distincts et séparés du système central merveux. Le SNHP ne peut être considéré comme un syndrome unique.

syndrome nerveux à haute pression
anésthétiques
anticonvulsants

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Biochimica et Biophysica Acta, 649 (1981) 125–128
Elsevier/North-Holland Biomedical Press

BBA Report

BBA 71545

CORRELATION OF GENERAL ANESTHETIC POTENCY WITH SOLUBILITY IN MEMBRANES

ANDREW S. JANOFF, MICHAEL J. PRINGLE and KEITH W. MILLER

Departments of Pharmacology and Anesthesia, Harvard Medical School and the Massachusetts General Hospital, Boston, MA 02114 (U.S.A.)

(Received March 24th, 1981)
(Revised manuscript received August 24th, 1981)

Key words: Anesthetic potency; Membrane solubility; Partition coefficient

Recently (Franks, N.P. and Lieb, W.R. (1978) Nature 274, 339–342) it has been claimed that the traditional correlation between anesthetic potency and vegetable oil solubility breaks down when the alkanols are compared to other volatile anesthetics. Lately, however, new information on the partitioning of anesthetics into lipid bilayers has become available. In this report the potency of twenty-one structurally diverse anesthetic agents is shown to correlate well with their ability to partition into phosphatidylcholine bilayers. Thus the original Meyer-Overton oil solubility hypothesis accomodates a wider range of anesthetics, including alkanols, volatile and gaseous agents, and barbiturates, when lipid bilayer solubility is substituted for oil solubility.

At the turn of the century Meyer and Overton, simultaneously but independently noted a correlation between anesthetic potency and olive oil solubility [1,2]. This correlation has stood the test of time and accurately predicts the potencies of gaseous and volatile anesthetics differing in potency by up to four orders of magnitude [3,4]. Meyer [5] assumed explicitly that olive oil was a model for the solubility properties of cellular lipids. However, later workers have not ruled out the possibility that the hydrophobic site modeled by olive oil is actually a suitable region in some protein. Recently theoretical arguments have been advanced which suggest that the anesthetic site might have substantial hydrophilic character [6]. Another study showed that the *n*-alkanols deviated systematically from other agents in the olive oil correlation but fitted a correlation using the octanol partition coefficient [7]. This study concluded that because of the polarity of octanol the physiological site of action of general anesthetics probably involves protein rather than the lipid region of some excitable membrane. On the other hand we note that the polar head group of the phosphatidylcholine molecule represents about 40% of its mass, so that it is not self-evident that the correlation with octanol solubility points uniquely to a protein as the site of action of anesthesia.

The most direct way of resolving the problem of interpreting the octanol correlation would be to measure the solubility of anesthetics in models that are of greater physiological relevance. The work of Seeman and co-workers [8] established a good correlation between the concentration at which many agents block nerves and their red cell/buffer partition coefficients, but since the red cell contains roughly equal quantities of lipid and protein by weight these data do not resolve the issue at hand. A better test would be provided by comparing general anesthetic potency with the partitioning behaviour of anesthetics into purely lipid bilayers. Such a test has only been performed with gaseous and volatile anesthetics [9] but not with the critical alkanols. However sufficient data have now accumulated from the efforts of

several groups to enable a much wider comparison to be made.

Partition coefficients for twenty one anesthetics in phosphatidylcholine bilayers are available in the literature. No other membrane has been examined in such detail. Although the phosphatidylcholine used by different workers varies with respect to the degree of saturation of the acyl chains (see legend to Fig. 1), this is known to have little effect on the partition coefficient [10,11]. Fig. 1 shows the correlation between the partitioning into phosphatidylcholine bilayers and anesthetic potency for twenty one agents, including alcohols, fluorocarbons, halocarbons, barbiturates, an inert gas and a ketone. Although the data are of heterogenous origin a remarkedly good correlation is obtained covering four orders of magnitude in dose for this widely diverse group of compounds. The line in Fig. 1 was fitted by the method of least squares which yielded a correlation coefficient, r, of -0.965 and a slope of -1.15 ± 0.072 (S.D.). This slope is not significantly different from -1 ($P > 0.05$) as is required by the theory. In contrast to the situation in olive oil [7], the alcohols (open circles) do not systematically deviate from this excellent correlation. In this respect the phosphatidylcholine solubility correlation is more successful than the traditional oil solubility correlation.

For membranes of other compositions there is less extensive partition coefficient data, but that available for thirteen agents covering three orders of magnitude in potency in phosphatidylcholine/cholesterol (2 : 1) bilayers and for thirteen agents covering three orders of magnitude in potency in the red cell membrane are to a first approximation consistent with our findings in phosphatidylcholine bilayers (see legend to Fig. 1 for details). Thus the presence or absence of cholesterol and/or protein apparently does not affect the correlation.

Although the correlation with lipid solubility in Fig. 1 is most persuasive, it still does not allow one to rule out the possibility that anesthesia may result from specific protein-anesthetic interactions. This model would be satisfied for instance if the appropriate lipid/protein partition coefficient were approximately one for all agents examined. While a direct approach to this problem is difficult because the exact site or sites of general anesthetic action remain undefined (for reviews see Refs. 4, 8 and 12), anesthetic-protein interactions might be expected to exhibit greater structural selectivity than anesthetic-lipid interactions. Thus examples of distinct structural requirements for anesthetic potency would provide a further test of theories of anesthesia.

The only case of apparent structural specificity for which the appropriate data is available at present is in fact consistent with the lipid hypothesis [13]. This is the case of the so called cut-off in potency in the alkanols (for a review see Ref. 12). As one ascends this series potency increases steadily until dodecanol, but tridecanol is only a partial anesthetic and tetradecanol and higher homologues exhibit no potency at all. Thus although it has been suggested that alkanols can specifically interact with the lipid bilayer [14] the sharp cut-off in potency turns out to be caused simply by a decrease in the membrane partition coefficient [13,15]. Fig. 2 shows that for the lower alcohols the maximum achievable concentrations in membranes (i.e. the product of saturated aqueous solubility and membrane buffer partition coefficient) is two orders of magnitude higher than that required to achieve anesthesia. This margin decreases slowly to decanol, after which successive additions of methylene groups cause a precipitious fall. By tetradecanol the maximum achievable concentration has become an order of magnitude lower than that required to cause anesthesia. Thus the loss of anesthetic potency that occurs in the higher members of the series is accurately predicted by the membrane aqueous phase partition coefficient. The presence or absence of protein apparently does not affect such predictions to a first approximation since they are valid both for biomembranes and lipid bilayers. Once again we cannot rule out that a similar correlation might be seen with some protein-alcohol interactions, but Figs. 1 and 2 taken together clearly restrict this probability.

Whether limitations in membrane solubility can also explain the cut-off in potency seen in the normal hydrocarbons cannot be assessed at present for lack of appropriate partition coefficient data but such an explanation appears likely [16].

The limited solubility of the longer chain alcohols calls into question many earlier spectroscopic studies which now appear to have been carried out under supersaturated conditions [13,17].

In conclusion, it should be evident that while the extension of the olive oil solubility correlation to

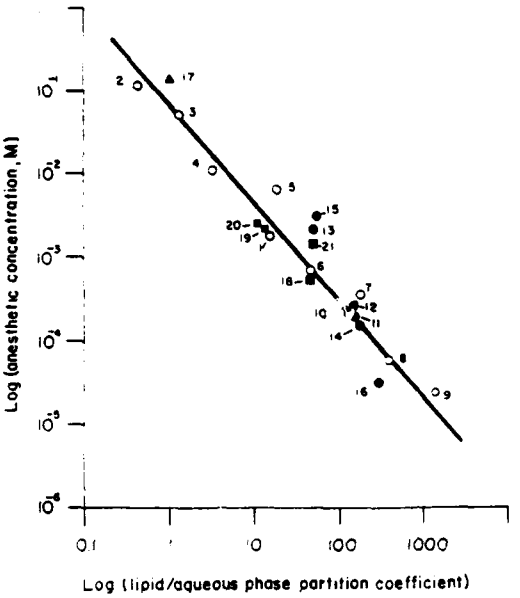

Fig. 1. For twenty one diverse agents there is a correlation between partitioning into phosphatidylcholine bilayers and the equilibrium anesthetic concentration (ED_{50}) in water bathing aquatic animals. The partition coefficient is defined as the equilibrium solute concentration per unit volume in the lipid phase divided by the equilibrium solute concentration in the aqueous phase. Symbols denote different animals; circles are tadpoles, squares are newts and triangles are frogs, all at room temperature. The alcohols are in open symbols, the rest are filled. The anesthetic data are from Pringle et al. [13] (compounds 2–4, 6 and 8); Meyer and Hemmi [18] (compounds 5, 7 and 9); Kita et al. [19] (compounds 1, 10 and 11); Miller and Ambalavanar (unpublished) (compounds 12 and 13); Lee-son et al. [20] (compounds 14–16); Meyer [21] (compound 17); Smith [22] (compounds 18–21). Partition coefficient data in dimyristoyl phosphatidylcholine: Katz and Diamond [23] (compounds 1–4 and 17). Partition coefficient data in egg phosphatidylcholine: Jain [24] (compounds 5–9); Colley and Metcalfe [25] (compound 1); Miller and Yu [26] (compound 14); Pang et al. [27] (compound 15); Korten et al. [28] (compound 16). Partition coefficient data in egg phosphatidylcholine: 4% phosphatidic acid: Smith et al. [9] (compounds 10–13 and 19–21); Miller et al. [10] (compound 18). Where tested dimyristoyl phosphatidylcholine, egg phosphatidylcholine and egg phosphatidylcholine: 4% phosphatidic acid bilayers have almost identical partition coefficients at room temperature [10,11,26]. Partition coefficients for the barbiturates were corrected in order to correspond to the pH (7.4) at which the anesthetic determinations were made. Anesthetics are referred to as follows: 1, benzyl alcohol; 2, ethanol; 3, propanol; 4, butanol; 5, pentanol; 6, hexanol; 7, heptanol; 8, octanol; 9, nonanol; 10, halothane; 11, methoxyflurane; 12, isoflurane; 13, fluroxene; 14, pentobarbital; 15, phenobarbital; 16, thiopental; 17, acetone; 18, cyclopropane; 19, xenon; 20, carbon tetrafluoride; 21, sulfur hexafluoride. Addition of 33 mol% cholesterol to the phosphatidylcholine bilayers did not change this correlation for those compounds for which data was available (compounds 1; 10–16; 18–21 and urethane). Anesthetic and phosphatidylcholine : cholesterol/aqueous phase partition coefficient data for urethane are available from Meyer [5] and Pang et al. [27], respectively.

The correlation was also not changed when partitioning into red cells was considered. Partition coefficient data: for compounds 1, 5–9 from Seeman [8]; for compound 10, Smith et al. [9]; for compound 14, 15, and 16, Korten et al. [28,29]; for compound 21, Power and Stegall [30]. Anesthetic and partition coefficient data for nitrogen is available from Smith [27] and Stegall [30], respectively.

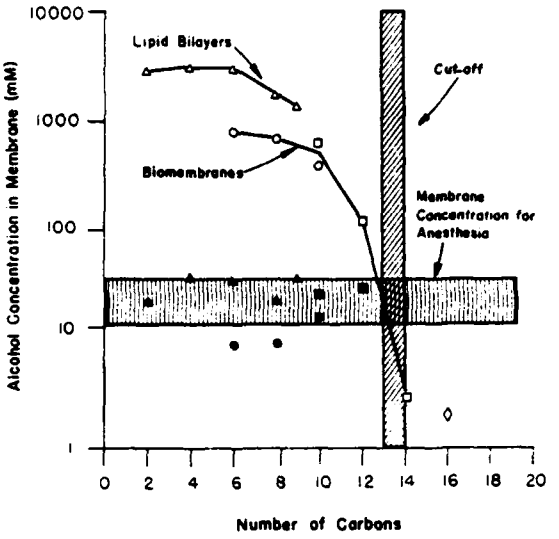

Fig. 2. The cut-off in potency for long chain alcohols is accounted for by membrane solubility. The partition coefficient for hexadecanol was determined after the method of Heap et al. [31]. The saturated aqueous concentration of nonanol is from Kinoshita et al. [32]. Sources for the rest of the data are given in Pringle et al. [13]. Symbols refer to different membranes. Triangles are phosphatidylcholine, diamond is egg phosphatidylcholine/cholesterol (2 : 1), circles are red cells and squares are intestinal brush border membranes. Open symbols are maximum alcohol concentration in membrane at saturation; filled symbols refer to anesthetic concentrations. Over the range of aqueous concentrations used, deviations from Henry's law are assumed to be minimal.

phospholipid bilayers provided here does not allow additional mechanistic insights, if Myer had been armed with this data many subsequent ambiguities in interpretation might have been avoided. Although the data presented here are consistent with the lipid hypothesis it remains possible that other models, as yet untested, might do as well.

This work was supported in part from a U.S. National Institute for General Medical Sciences Grant (GM-25911) and in part by joint funding from the U.S. Office of Naval Research and the Naval Medical Research and Development Command through O.N.R. contract N 00014-756-0727. K.W.M. is a Research Career Development Awardee of the National Institute of General Medical Sciences (GM 00199).

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THE EFFECTS OF HIGH PRESSURES OF INERT GASES ON CHOLINERGIC RECEPTOR BINDING AND FUNCTION

J. F. Sauter, L. Braswell, P. Wankowicz, and K. W. Miller

The ability of pressure to excite animals has been known for about a hundred years (1). Nonetheless, little detailed progress has been made towards understanding the underlying events. Recently a number of workers have begun to explore the electrophysiological changes which occur when pressure is raised. (These studies have been reviewed recently by Wann and Macdonald [2].) In addition to these pioneering studies, neurochemical data will be required if a complete description is to be arrived at. Such studies can directly answer questions such as, What is the effect of pressure on neurotransmitter release and binding, on cyclase activation, and so on?

One advantage of such studies would be that they can be readily applied to the central nervous system of animals. Because many of the manifestations of pressure excitability are thought to be central in origin, and because electrophysiological studies in the brain are difficult, one would expect a neurochemical approach to be particularly fruitful. Nonetheless, few such studies have been undertaken (3) and little has been done towards developing techniques. In this paper we examine the effects of pressure on [^{3}H]-acetylcholine binding to the acetylcholine receptor using apparatus we have developed for performing filtration assays. Although we have applied this technique here to a peripheral synapse (to be described), with minor adaptions it could be used to study a wide variety of central receptors.

For these studies we chose the nicotinic cholinergic receptors that can be isolated from the electroplaque of certain electric fish. This synaptic membrane can be obtained in high yield and specific activity and offers the best model for studies on the postsynaptic mechanisms of action of pressure. Furthermore,

because electric tissue was developed from muscle cells, this synapse has many of the characteristics of the neuromuscular junction. Thus, the most powerful electrophysiological and biochemical techniques can both be brought to bear on the problem.

The effects of pressure on the neuromuscular junction have been studied. Campenot (7) found that end-plate potentials in lobster and crab neuromuscular junction were depressed by hydrostatic pressure (200 atm). In the crab this effect was overcome when higher stimulation frequencies were employed. Kendig and Cohen (4) showed that 137 atm of helium had no effect on the indirectly elicited electromyogram (EMG) of rat diaphragm unless the calcium concentration in the bath was lowered. They too believed pressure was exerting a presynaptic effect. More recently Wann et al. (5) have made more detailed studies of the frog neuromuscular junction. They found that hydrostatic pressure caused a marked reduction in miniature end-plate current (MEPC) frequency at 102 atm; a finding that indicates a reduction in transmitter release. At higher pressures the decay phase of the MEPC was lengthened from a control value of 1.5 ms to a value of 2.1 ms at 153 atm. Their results suggested that the rate process governing decay has an activation volume of 56 mL mol^{-1}.

METHODS

Preparation of Acetylcholine Receptor-Rich Membranes

The method outlined here was adapted from Cohen et al. (7). Briefly, 100 g of fresh skinned electric tissue, dissected from a chilled *Torpedo californica* immediately upon receipt, is minced and added to 200 mL of cold azide water, homogenized on a Vertis apparatus at full speed for 3 min, briefly sonicated and centrifuged at 5,000 xg for 10 min. The supernatant is filtered and centrifuged at 15,000 xg for 90 min. This pellet is resuspended in distilled water and centrifuged through a 1.08 M sucrose cushion at 80,000 xg for 90 min; this step removes much acetylcholinesterase. The soft pellets are resuspended in water and the hard pellet discarded. The resuspended pellet is placed on a sucrose gradient, consisting of equal volumes of 1.5, 1.4, 1.3, 1.2, 1.1, and 0.8 M sucrose and centrifuged at 80,000 xg for 4½ h. Fractions of 1 mL are fractions collected from the bottom and assayed for acetylcholine receptors (AchR) using a tritiated snake toxin (a kind gift to us from J.B. Cohen), acetylcholinesterase (AchE), protein, and sucrose.

The sucrose gradient profile from an experiment is shown in Fig. 1. The specific activity in the pooled receptor peak is usually 1–3 μmol AchR/g protein. Receptor is stored at 4°C as collected. For critical use it is used within 3 weeks, but activity is usually present even after several months. The membranes may also be frozen and later thawed on ice as required.

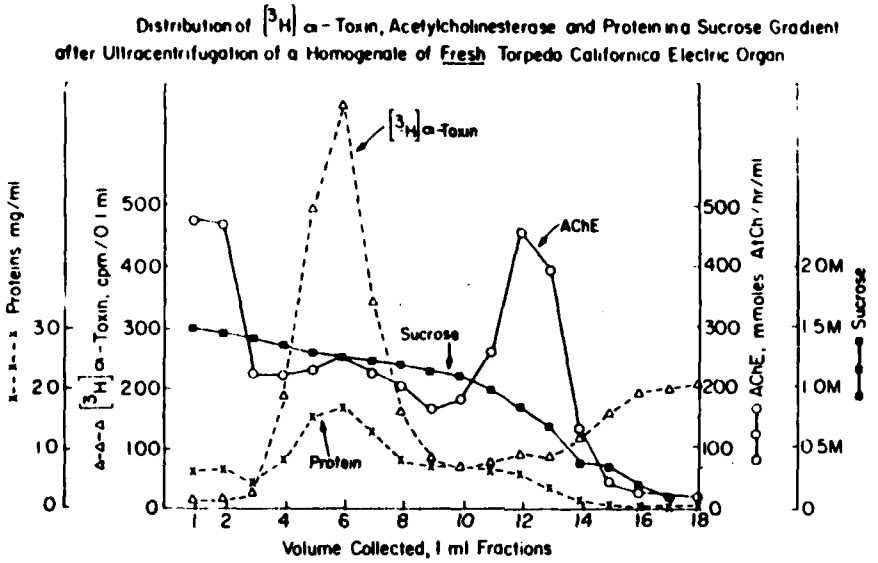

Fig. 1 Profile of a sucrose density gradient used in the final step of the isolation of acetylcholine receptor-rich membranes from *Torpedo californica*. The α-toxin marks the position of the receptor containing membranes

Binding of [^{3}H]-Acetylcholine to Membranes

The equilibrium binding of [^{3}H]-acetylcholine (Amersham-Searle, Arlington Heights, IL) to receptor containing membranes was determined by filter assay. Whatman GFF glass fiber filters are used to separate the membranes from the buffer once the equilibrium is attained. The amount of acetylcholine bound is determined from the difference between total counts and the counts in the filtrate. The difference between the amount bound in the presence and absence of excess α-bungarotoxin is defined as *specific binding*. This correction is only a few percent of the bound ligand. Because AchE is present (Fig. 1), its activity must be inhibited with di-isopropylfuorophosphate (DFP). Typically, 1.2 mL of AchR stock solution (3μM, in sucrose) is diluted to 13.5 mL with Torpedo Ringer (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 5 mM sodium phosphate, pH 7) and mixed with 1.5 mL of 10^{-3} M DFP. Controls show these conditions block all the AchE activity after 30 min incubation. Finally, this preparation is split into aliquots and diluted to give the final AchR concentrations noted in results. These are usually in the range of 20–50 nM. [^{3}H]-acetylcholine is allowed to equilibrate with the membranes for 30 min before filtration.

The kinetics of acetylcholine binding to its receptor were determined similarly except that equal aliquots of receptor suspension and of [^{3}H]-acetylcholine are mixed rapidly at zero time and the amount of binding determined as a function of time.

Filtration Assays at Pressure

Work in hyperbaric gaseous environments is carried out in an 8-inch diameter steel chamber capable of working to 300 atm. Twelve filtration units are mounted on a turntable in the chamber. They may be moved in turn to a position where they may be remotely connected to the outside of the chamber thus providing suction. This process is operated and timed by a microprocessor that gives smooth, reproducible filtering. Solutions to be filtered may be placed above the filters on diaphragms that rupture at the commencement of filtration, or may be delivered from motor-driven syringes. Incubating mixtures may be stirred magnetically. A more detailed description of this apparatus has been given (8).

RESULTS

We first determined whether exposure to 300 atm of helium caused irreversible effects on the receptor containing membranes. The incubation wells of the 12 filtration units were filled with receptors and [^{3}H]-acetylcholine. Six units were filtered in the chamber at 10 atm of helium (A small amount of pressure is necessary to ensure filtration. The remaining receptors were compressed to 300 atm, held for 30 min, and slowly decompressed to 10 atm, where they were filtered. The amount of acetylcholine bound was the same in both sets of filters.

We next examined the effects of helium pressure on binding. A simple assay was used in which the incubation mixture contained sufficient [^{3}H] acetylcholine to half-saturate the receptor sites. The ratio of the concentration of bound acetylcholine to that of free acetylcholine changed from 1.0 to 0.71 at 300 atm. A similar effect was observed with the antagonist [^{3}H]-d-tubocurarine. The cause of this decrease could either be loss of receptor sites or a reduction in affinity, that is, an increase in dissociation constant, K_d. Accordingly, receptor containing membranes were equilibrated with a wide range of [^{3}H]-acetylcholine concentrations. The receptor sites were progressively saturated as the [^{3}H]-acetylcholine concentration was raised. On the bench filtration assays typically yield binding curves that exhibit positive co-operativity with a Hill coefficient of about 1.5 and a Hill dissociation constant of 20 ± 9 nM, where the variation reflects the difference between different batches of receptor and not the experimental error. In one experiment shown in Fig. 2, the Hill dissociation constant, K_d, decreased from 16 ± 2.2 nM at 5 atm to 23 ± 3.2 nM at 275 atm of helium, whereas the Hill coefficient, n_H, which was 1.5, did not change. Two such experiments have been performed up to this time and the mean ratio of the K_d at high pressure to that at low pressure was 2.6 ± 0.7. The binding of acetylcholine is a complex process, but if we make

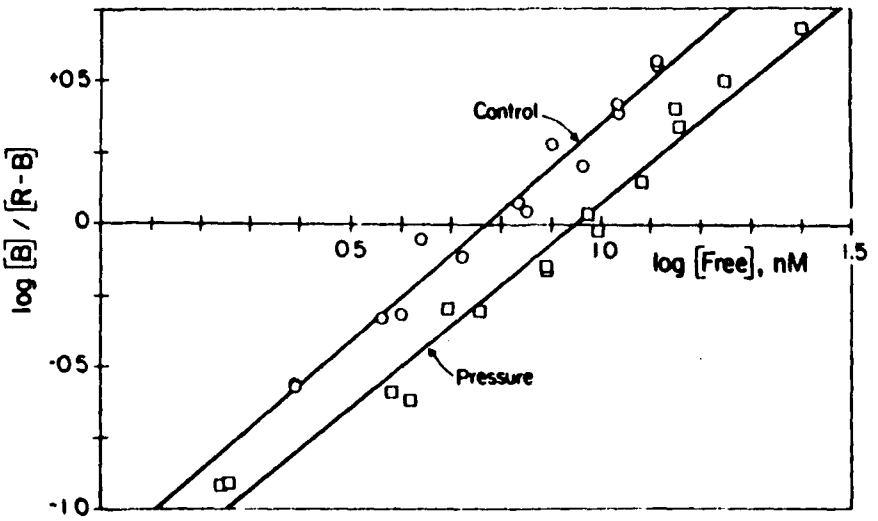

Fig. 2. Hill plot of the effect of pressure (275 atm) on specific [^{3}H]-acetylcholine binding to *Torpedo* membranes. B is bound acetylcholine and R is the total receptor binding sites (~30 nM). Pressure decreases the binding affinity without changing the slope.

the simplifying assumption that the K_d represents an equilibrium we may calculate thermodynamically that a volume change of 80 ± 30 mL/mol is involved in the overall processes involved in acetylcholine binding.

To understand the origins of the effects of pressure on acetylcholine binding, detailed kinetic experiments will be required. These are technically very demanding and only preliminary results are available at pressure. The binding of [^{3}H]-acetylcholine is biphasic. An initial rapid binding step, which is too fast to follow experimentally, is followed by a slow phase with a time constant of a few minutes (Fig. 3). This second step can be characterized kinetically. The observed rate constant is usually in the range 5-8 × 10 ^{3}s $^{-1}$. Figure 3 shows a pair of experiments conducted within a few days of each other on the same membrane preparation. The rate constant at 300 atm is decreased relative to that at 10 atm by by a factor of 1.6, but the value at pressure is close to the range of values generally obtained on the bench. Thus, further work will be required to establish the significance of the change seen here. In addition, if the slow phase extrapolated back to zero time, the intercept yields an estimate of the amplitude of the fast phase. This is slightly decreased by pressure.

It is of interest to ask if the other diving gases cause effects similar to those of helium. Our preliminary data show that in fact they cause changes in the opposite direction. After 30 min stirring to equilibrate with the gaseous atmosphere, both nitrous oxide (3.5 and 6 atm) and argon (100 atm) caused increases in [^{3}H]-acetylcholine binding (Table I).

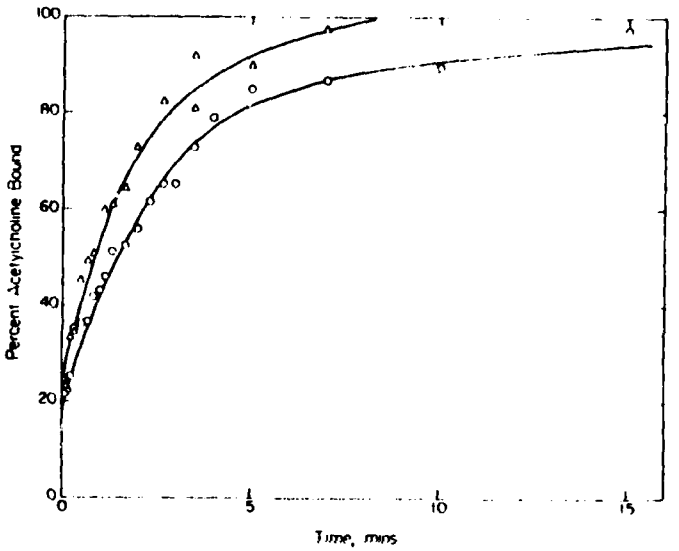

Fig. 3. The kinetics of binding of [^{3}H] acetylcholine to *Torpedo californica* receptor rich membranes. Both experiments were carried out in the pressure chamber. Δ, 5 atm of helium. [AchR] = 25 nM, [Ach] = 48 nM; rate constant = 7.7 ± 0.84 × 10 ^{3}s $^{-1}$. ○, 300 atm of helium. [AchR] = 27 nM, [Ach] = 50 nM; rate constant = 4.7 ± 0.17 × 10 ^{3}s $^{-1}$.

TABLE I

Change in [^{3}H]-Acetylcholine Binding to *Torpedo californica* Receptor-Rich Membranes After Exposure to Inert Gases for 30 Min

Gas	Pressure (ATA)	[Bound Acetylcholine]/[Free Acetylcholine]
Air	1	1.00
Helium	300	0.75
Argon	100	1.37
Nitrous Oxide	3.5	1.25
Nitrous Oxide	6.0	2.04

DISCUSSION

Although this research is ongoing and much of the quantitative data are still incomplete, certain qualitative conclusions stand out. First, the effects of inert gases on the binding of [^{3}H]-acetylcholine to its receptor follow a similar pattern to that observed in the behavior of animals. In animals helium at pressure causes excitation while argon and nitrous oxide cause depression and general anesthesia (9,10). The sign, or direction, of the effect changes when helium is compared to the other gases. Similarly, the direction of the effect on

acetylcholine binding changes. Binding decreases with helium, whereas it increases with argon and nitrous oxide. When the underlying mechanisms of these effects are finally established it is possible that they will bear some similarities to the events occurring in vivo.

The effects of helium pressure we have investigated in some detail. The binding of acetylcholine to its receptor is a complex process, which may be represented approximately as

$$\begin{array}{ccc} A + R & \rightleftharpoons & AR_o \\ \| & & \| \\ A + R' & \rightleftharpoons & AR' \end{array}$$

where A is acetylcholine, R is resting receptor, R_o is receptor associated with an open channel, and R′ is desensitized (that is, inactive) receptor (11). Thus, the overall equilibrium measured for acetylcholine binding represents the sum of several processes (12,13). Our results show that pressure changes the overall dissociation constant without changing the cooperativity of binding (Hill coefficient). Such cooperativity suggests that more than one acetylcholine molecule binds to each receptor molecule. Available data suggest there are two acetylcholine binding sites per receptor complex (14,15). Thus, pressure has no effects on the allosteric interactions between these sites.

To define which steps in the cyclic scheme above are effected by pressure requires more detailed kinetic studies. Our studies up to this time (Fig. 3) utilize the fact that R binds acetylcholine with much lower affinity than R′ and also that the vertical equilibria are slow and the horizontal ones very fast. Therefore, the initial concentration of acetylcholine can be adjusted so that initially all R′ is bound rapidly but no R binds. Then, as R⟶R′, attempting to re-establish the equilibrium, more acetylcholine will bind at a rate representing the rate of formation of R′. This is the situation shown Fig. 3. This single experiment, which requires confirmation, suggests that the resting amount of R′ is slightly reduced by pressure while, consistently, the rate of R⟶R′ is reduced. These effects are small but, if confirmed, would suggest that pressure does not act only at this step because the change in overall dissociation constant is too large. In particular, one might expect that if the binding of acetylcholine (a cation) to R′ involves any charge neutralization, a positive volume change would occur because of the release of electrostricted water (16). The dissociation constant for this step would also be increased. If this turns out to be true, then pressure acts both on R⟶R′ and A + R′⟶AR′.

It is important to realize that the most critical reason why our results on the effects of pressure are tentative is that the effects in the pressure range studied (up to 300 atm) are fairly small and therefore require accurate quantitation. We can say from Fig. 3 that the conformational change R⟶R′ is affected little by pressure, even though several more experiments will be required before we can be certain how small the effect is. The rate of decay of

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MEPC's in frog is also decreased only 1.4 fold by 154 atm (5). This can approximately be taken as a measure of the effects of pressure on $AR_n \rightarrow AR'$. Our results for $R \rightarrow R'$ yield an activation volume of 45 mL/mol, whereas the latter data for $AR_n \rightarrow AR'$ yield 56 mL/mol. Thus, both conformation changes in this membrane protein are associated with similar volume changes.

We have not yet investigated in detail how the effects of other gases, such as argon, are brought about. What we know so far suggests they act in a similar manner to the volatile anesthetics (17,18). These agents increase acetylcholine binding and can increase the proportion of R' in the resting state.

This work clearly demonstrates that biochemical techniques can be applied to the study of ligand binding to postsynaptic membranes in hyperbaric gaseous environments. The data reported here concern a peripheral synapse from a fish adapted to moderate pressures. Nonetheless, it exhibits many of the expected properties. It thus serves as a useful model for the detailed elucidation of the underlying mechanisms. These techniques can also be applied, though not in such a detailed manner, to central mammalian synaptic membranes. It remains to be seen whether the effects of pressure on these will be more profound.

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PATTERNS OF INTERACTION OF THE EFFECTS OF THE LIGHT METABOLICALLY INERT GASES WITH THOSE OF HYDROSTATIC PRESSURE AS SUCH - A REVIEW

BY:

R. W. BRAUER[1], P. M. HOGAN[2], M. HUGON[3]

A. G. MACDONALD[4], AND K. W. MILLER[5]

[1]INSTITUTE OF MARINE BIOMEDICAL RESEARCH
UNIVERSITY OF NORTH CAROLINA AT WILMINGTON, N.C., USA

[2]DEPARTMENT OF PHYSIOLOGY
STATE UNIVERSITY OF N.Y. AT BUFFALO, N.Y., USA

[3]G.I.S PHYSIOLOGIE HYPERBARE
CNRS
MARSEILLE, FRANCE

[4]DEPARTMENT OF PHYSIOLOGY
MARISCHAL COLLEGE, ABERDEEN UNIVERSITY
ABERDEEN, SCOTLAND

[5]DEPARTMENT OF PHARMACOLOGY AND ANESTHESIA
HARVARD UNIVERSITY MEDICAL SCHOOL
BOSTON, MASS., USA

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Medical Center
Philadelphia, PA 19104

Dr. Claes E. G. Lundgren
Department of Physiology
120 Sherman Hall
School of Medicine & Dentistry
SUNY at Buffalo
Buffalo, NY 14214

Dr. Edmundo Ashkar
Director, Hyperbaric and
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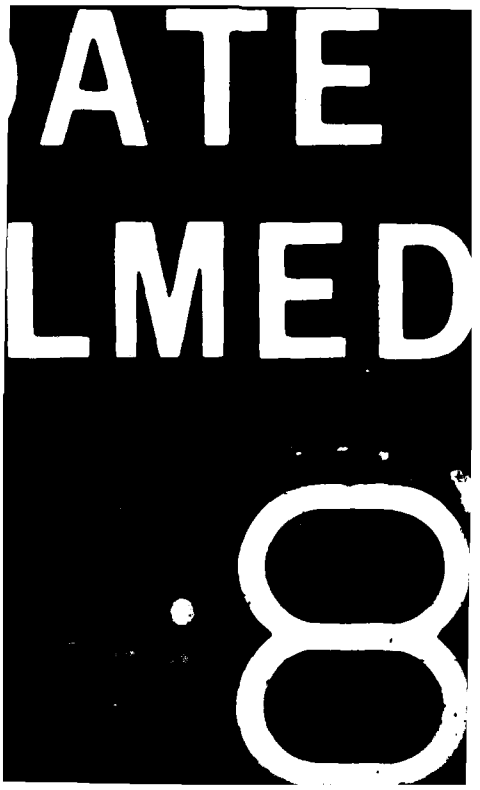
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